Investigating the safety of meat co-products: microbiology aspect

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Linakshi Weerakoon
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Abstract

Meat co-products (offal) are rich in protein and essential nutrients and have been consumed as delicacies worldwide. China, New Zealand's largest red meat export market is a country where offal dishes are frequently consumed. As foodborne diseases are a major challenge faced by Chinese consumers, it is important to ensure the quality and safety of offal consumed in China. The objectives of the study were; firstly to investigate the presence of *E. coli*/ coliforms, *Campylobacter jejuni*, Salmonella, *Clostridium perfringens*, *Listeria monocytogenes and* determine the aerobic plate count (APC) of sheep offal (testes, skirt, liver, tripe, kidney, heart, tail and pizzle) purchased from New Zealand and China using conventional microbiology enumeration methods. Secondly, the distribution of microbial populations present in the sheep offal were investigated using metagenomics. Thirdly, the presence of mycotoxins, aflatoxin B_1 (AFB_1), deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin and ochratoxin A (OTA) in sheep offal were investigated. Lastly, the decontamination efficiency of chitosan on meat co-products was investigated. *Campylobacter jejuni*, *Salmonella*, *Clostridium perfringens*, *Listeria monocytogenes* were not present in any of the sheep offal. APC counts obtained for testes, skirt, liver, tripe , kidney, heart, tail and pizzle were 1.85 ± 0.58, 1.65 ± 0.53,1.41 ± 0.28, 1.61± 0.51,1.53 ±0.97, 2.16 ± 0.18 and 2.35 ± 0.46 log CFU/g, respectively for the New Zealand sheep offal and 6.27 ± 0.25, 6.04± 1.53, 6.36 ± 0.72, 5.70 ± 0.92, 7.56 ± 0.58, 7.41 ± 0.56, 7.41 ± 0.45 and 7.44± 1.11 log CFU/g, respectively for the Chinese sheep offal. Coliforms were not present in the New Zealand sheep offal samples. However, in the Chinese sheep offal coliform counts of 4.67 ± 0.96, 5.10 ± 0.60 5.01 ± 1.02, 4.77 ± 0.52, 7.12 ± 0.16 log CFU/g were found to be present in testes, skirt, liver, tripe, and kidney respectively. There was no *E. coli* present in any of the sheep offal samples. The metagenomic analysis revealed that Proteobacteria, Fermicutes, Actinobacteria, Bacteroidetes and Fusobacteria were the predominant phyla in the investigated sheep offal. However, different abundance levels of these phyla were observed between the samples from the two countries. Community abundance at Genus level indicated the presence of psychrotrophic foodborne bacteria.

All of the New Zealand offal were positive for AFB_1. The highest and lowest AFB_1 concentrations were present in skirt (13.77±7.55 μg/kg) and liver (0.88±0.76 μg/kg) samples respectively. In the Chinese samples only pizzle, kidney, tripe and liver were positive for AFB_1. The highest concentration was detected in liver (0.83 μg/kg) and the lowest was in pizzle (0.51±0.15 μg/kg). T-2 toxin was only present in the kidney, heart and pizzle samples of New Zealand. The highest and lowest concentrations were present in skirt (3.512 μg/kg) and heart (1.37±0.15 μg/kg) respectively. In the Chinese samples T-2 toxin was present only in skirt

samples (0.03±0.01 μg/kg). All offal types from New Zealand were positive for DON except the pizzle and tripe samples. In the Chinese samples, only liver, tripe and kidney were positive for DON. OTA and ZEA were not present in any of the sheep offal from either of the countries.

Non-irradiated crab chitosan was used to treat sheep tripe samples inoculated with *S. aureus* and *E. coli* O157:H7. The *E. coli* count on tripe was significantly reduced ($p<0.05$) to 4.31 log CFU/g and 3.88 log CFU/g when treated with chitosan at 0.31 mg/ml and 1.25 mg/ml respectively from an initial count of 5.30 log CFU/g. A significant reduction ($p<0.05$) in *E. coli* count was observed only with the 1.25 mg/ml treatment. The *S. aureus* count on the tripe samples was found to be reduced ($p<0.05$) to 4.695 log CFU/g and 3.710 log CFU/g when treated with chitosan at 0.31 mg/ml and 1.25 mg/ml, respectively compared to the initial *S. aureus* count (5.34 log CFU/g).

Keywords: Meat co-products, Foodborne pathogens, Mycotoxins, Metagenomics, Chitosan

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List of Abbreviations

AD	Acetylation degree
AF	Aflatoxin
ANZFA	Australia New Zealand Food Authority
APC	Aerobic plate count
ASC	Acidified sodium chloride
^{0}C	Celsius
C. coli	*Campylobacter coli*
CFU/ml	Colony forming units per millilitre
C. jejuni	*Campylobacter jejuni*
Cm	Centimetre
CPC	Cetylpyridinum chloride
C. perfringens	*Clostridium perfringens*
DDA	Deacetylation degree
DON	Deoxynivalenol
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FDA	Food and Drug Administration
FIT	Federal inspection type
µg/kg	Microgram per kilogram
g/mol	Gram per mol
GRAS	Generally recognized as safe
h	Hours
HACCP	Hazard analysis critical control points
HMWC	High molecular weight chitosan
HPP	High pressure processing
IMSF	International Commission on Microbiological Specifications for Food
J/cm^2	Joules per square centimetre
kDa	Kilodalton
kGy	Kilogray
kPa	Kilopascal
L	Litre
LA	Lactic acid

L. monocytogenes	*Listeria monocytogenes*
log CFU/cm^2	Log colony forming units per square centimetre
log CFU/g	Log colony forming units per gram
LPS	Lipopolysaccharide
MAP	Modified atmosphere packaging
MBC	Minimal bactericidal count
MDA	Malondialdehyde
mg/g	Milligram per gram
mg/ml	Milligrams per millilitre
MHB	Mueller Hinton broth
MIC	Minimal inhibitory concentration
mm	Millimetre
MPa	Megapascal
Mw	Molecular weight
ng/g	Nanogram per gram
OTA	Ochratoxin A
PL	Pulsed light
ppm	Parts per million
PW	Peptone water
QAC	Quarternary ammonium compound
RTE	Ready-to-eat
S. aureus	*Staphylococcus aureus*
S. Typhi	*Salmonella Typhimurium*
TAB	Total aerobic bacteria
UK	United Kingdom
USDA	United States Department of Agriculture
UV	Ultraviolet
ZEA	Zearalenones

Chapter 1: Introduction

Offal or edible meat co-products are defined as "any edible product other than red or white meat muscles obtained from slaughtered animals and birds" (Lynch et al., 2018). These co-products could be a solution for the global demand for protein rich food. The Food and Agriculture Organisation (FAO) has reported that pork, lamb and beef offal are potentially rich sources of lysine, methionine and tryptophan, which may be limited in poor quality protein sources (Mullen et al., 2017). Offal is not only rich in protein, but in minerals and vitamins as well. For instance, liver is a rich source of vitamins A, B12 and folate. Minerals such as iron and copper are known to be present in high contents in almost all offal (except tripe) in comparison to lean meat (Lynch et al., 2018).

Certain offal dishes such as beef tongue, lamb kidney and tripe from both species are much appreciated by different cultures and are considered to be delicacies. For example, the "Mondongo" dish from Cuba and Mexico is a popular preparation of tripe (Nollet et al., 2011; Lynch et al., 2018). In some countries such as Korea, beef tripe and liver are commonly consumed raw (Jeong et al., 2017). The rich nutrient composition, high moisture content and pH (5.5 - 6.5) may facilitate the growth of a large range of microorganisms on meat (Nychas et al., 2008; Doulgeraki et al., 2018) and this may explain the association between foodborne disease incidence and the consumption of undercooked offal dishes which have been reported in these studies. In Australia, a case of salmonellosis was found to be linked to the consumption of contaminated lamb liver (Hess at al., 2008) and in the United Kingdom (UK) outbreaks of campylobacteriosis and salmonellosis have been linked to the consumption of chicken liver (Little et al., 2010; Merritt et al., 2011). Therefore, ensuring high levels of safety and quality in meat co-products is of utmost importance.

China, a country with high levels of offal consumption, imports sheep offal such as heart, tripe, liver, kidney among others, from New Zealand and other meat-producing countries. China is New Zealand's second largest market for sheep and beef exports (Shepherd, 2017). However, China is challenged by foodborne diseases and studies from 2012 have revealed that 56.1% of food poisoning outbreaks were caused by microorganisms (Pei et al., 2015).

A total of 209 million foodborne cases were reported in the period of 2010-2011 in China (Wu et al., 2018). A review published in 2013 revealed that in China 52.2% of foodborne diseases in humans were due to the consumption of animal-based food (Paudyal et al., 2016). *Salmonella*, *Listeria monocytogenes*, *Campylobacter* and *E. coli* O157:H7 are the main

pathogens causing health issues accountable for more than 90% of estimated food-related deaths (Scallan et al., 2011). Major foodborne pathogens have been detected in meat products in China and New Zealand. For instance, *L. monocytogenes* was found in fresh pork, fresh beef, fresh poultry, frozen raw meats, fresh mutton and ready-to-eat (RTE) meats in China at 11.3%, 9.1%, 7.2%, 7.2% ,5.4% and 3.2% of the tested samples, respectively (Liu et al., 2020). RTE meats from New Zealand also were found to contain *L. monocytogenes* at prevalence of 4.3% (Cornelius et al., 2008). *Salmonella* has also been isolated from retail meat products (pork, chicken, beef mutton) in China and New Zealand (Zhang et al, 2018; Wong et al., 2007). This information has motivated the investigation of the safety of sheep offal in New Zealand and China in the present study.

Salmonella spp, *E. coli* and *L. monocytogenes* are organisms tested for in export meat and meat products to ensure safety (Salih et al., 2019). In addition, aerobic plate counts (APCs) and coliforms counts will also be detected. APC serves as a general indicator of microbial contamination. Faecal contamination of food is indicated by counts of coliforms and the presence of *E. coli* in food (Im et al., 2016). Meat samples with APC counts exceeding 7 log CFU/g are considered to be unfit for human consumption according to the International Commission on Microbiological Specifications for Foods (ICMSF) criteria (Im et al., 2016).

Meat and poultry products are biologically diverse and may contain thousands of bacteria, virus and fungi (Weinroth et al., 2019). Within this community, each organism has its own genome, while some genes may be specialized/specific to specific microorganisms and some are ubiquitous. Investigating community-wide interactions rather than separate bacterial groups can provide a much clearer picture of the microbial distribution in a sample. However, certain bacteria present within meat may be difficult to culture in a laboratory. Since these uncultivable bacteria may present a challenge for the safety and quality of meat products researchers have introduced culture-free approaches such as metagenomics to investigate microbial populations (Weinroth et al., 2019). Metagenomic analysis provides an unbiased view of a microbial community's metabolic potential and its structure (Hugenholtz & Tyson, 2008). In this method, microbial DNA is isolated from a sample and directly sequenced followed by suitable bioinformatics analyses to investigate the functional traits of microorganisms in different environments (Coughlan et al., 2015). Amplicon sequencing using 16s rRNA gene sequencing is one of the strategies used in metagenomics (Rausch et al., 2019). This is an application where the concept of 16S rRNA sequence-based classification can be used to characterize biodiversity and to investigate the ecological characteristics of any sample. The ability to compare with detailed and well-curated taxonomic databases is a benefit of this sequencing technique

(Christensen et al., 2018). In the present study, the distribution of microbial populations present in sheep offal purchased from New Zealand and China will be investigated using 16S rRNA sequence-based metagenomics.

Mycotoxins are a group of heterogeneous secondary metabolites produced by fungi (mainly by genera *Aspergillus*, *Penicillium* and *Fusarium*). Due to their toxicity and heat stability, they are extremely hazardous to both humans and animals. The most important mycotoxins on the basis of their occurrence and toxicity are aflatoxins (AFs), trichothecenes (T-2 toxin and deoxynivalenol), ochratoxin A (OTA) and zearalenones (ZEAs) (Iqbal et al., 2014). For example, consumption of ZEA contaminated food may impair reproduction in females due to its relationship with human estrogenic activity (Adegbeye et al., 2020).

Mycotoxins may pass on to meat if the animals consume mycotoxin contaminated feed (Gareis & Scheuer, 2000). Though mycotoxins could be excreted in, milk, faeces or urine it also could be accumulated in meat and visceral organs that are consumed by consumers, exposing them to mycotoxins' negative effects (Adegbeye et al., 2020). Mycotoxin contaminated food are of great concern due to their ability to resist sterilization and classical cooking processes (Park, 2002; Bailly & Guerre, 2009). Ruminants are known to be more resistant to mycotoxin accumulation than monogastric animals due to the presence of rumen protozoa capable of degrading T-2 toxin, OTA, and ZEA (Adegbeye et al., 2020). Yet, studies have reported the presence of mycotoxins in ruminant organs. For example, AFB_1 was found to be present in beef livers and kidneys in the ranges of 0.00-20.00 and 3.80-24.00 µg/kg, respectively (Hassan et al., 2014). In a study from Iran it was reported that buffalo liver contained ZEA at a concentration range of 0.10-4.34 ng/g (Mahmoudi, 2014). Due to the limited studies on mycotoxin contents in New Zealand sheep organs, the present study investigated the presence of AFB_1, deoxynivalenol (DON), ZEA, T-2 and OTA in different sheep offal from New Zealand and compared it with comparable samples from China.

Prior to slaughter, muscle tissue of healthy animals is considered sterile. However, lymph nodes and surfaces can be exposed to gastrointestinal tract, external hide or fleece during the slaughter process and may lead to extensive contamination (Song et al., 2018). This contamination alongside plant possible cross contamination could serve as a source of contamination for meat during slaughtering and processing. Therefore, various approaches have been implemented to minimize potential carcass contamination (Sofos & Smith, 1998).

At present, physical and chemical decontamination techniques of meat are used by the meat industry. In the physical decontamination, microorganisms are eliminated without the use of

antimicrobial additives or products that affect microbial metabolism. Water spray washing, trimming and steam pasteurization are some of the techniques used (Bacon, 2005). Chemicals of various degrees of effectiveness such as chlorine, organic acids and ozone are utilized to destroy pathogenic bacteria present on meat during the chemical decontamination. However, these techniques may at times be ineffective if bacteria have been attached to the meat for a long time (Bacon, 2005). In addition to these techniques, there are emerging techniques for meat decontamination such as pulsed light, irradiation and high pressure processing (HPP) that are being investigated (Tomasevic et al. 2019; Nam et al., 2017).

Some of these techniques have been found to alter the sensory aspect of meat. Clariana et al. (2011) reported increased odour and brightness in dry cured ham subjected to HPP at 600 MPa. Irradiation has been reported to facilitate lipid oxidation in meat giving rise to off-odours (Nam et al., 2017). Due to these negative outcomes as well as the increased demand for minimally processed chemical free food, the effectiveness of natural antimicrobial agents against meat decontamination is currently being investigated (Hyldgaard et al., 2012; Jayasena & Jo, 2013). Chitosan, which is chitin's deacetylated form (Chung et al., 2003), has received increasing attention due to its antibacterial properties against foodborne pathogens (Cao et al., 2009; No et al., 2002; Govaris et al., 2010). Kim et al. (2007) reported significant reductions in *Escherichia coli* (*E. coli*) and *Salmonella enterica* Typhi (*S. enterica* Typhi) by using chitosan on chicken meat that was artificially inoculated with the two strains. Furthermore, when Harbin sausages were treated with an edible chitosan coating the total aerobic bacteria (TAB) were found to decrease to meet China's national standard for TAB (5 log CFU/g) (Dong et al., 2020). Therefore, in the present study the effectiveness of chitosan as a decontamination agent for meat was investigated.

1.1 Objectives

1.1.1 Investigate and compare the presence of major foodborne pathogens (APC, *E. coli*/ coliforms, *Campylobacter jejuni*, *Salmonella* spp., *Clostridium perfringens* and *Listeria monocytogenes*) in sheep offal purchased in New Zealand and China using conventional microbiology enumeration methods.

1.1.2 Investigate and compare the distribution of microbial populations present in sheep offal purchased in New Zealand and China using metagenomics technique.

1.1.3 Investigate the presence of AFB_1, DON, ZEA, T-2 and OTA in sheep offal purchased in New Zealand and China.

1.1.4 Investigate the antibacterial effect of chitosan as a decontamination agent for meat co-products

Chapter 2: Literature Review

2.1 Meat Offal

Meat is a great source of essential nutrients such as essential amino acids, minerals (iron and zinc) and vitamin B group. Due to its high nutrient density, meat plays an important role in increasing food security and reduce malnutrition. Around the world, the demand for meat has increased massively in the last 20 years (Alao et al., 2017). This has led to a rapid increase in livestock production, which leads to the generation of high quantities of animal by-products/co-products (Alao et al., 2017). The parts, other than the carcass, of slaughtered animals obtained after dressing are generally termed "animal by-products/ co-products". These could be edible (fit for human consumption) or inedible. For instance, liver, kidney, tongue and heart are edible and rich in essential nutrients (vitamins A, B12, folate, iron, copper, etc.), which may be limited in meat and meat products. Therefore, these organs could serve as a solution for malnutrition in developing countries (Alao et al., 2017). Inedible co-products such as bones, hides and skin, feathers, hooves, and horns, are generally processed into valuable biotechnological products for livestock and human. Bones are reprocessed into livestock feed whereas skin and feathers are processed and used in upholstery, leather and textile industry (Alao et al., 2017).

The edibility of a by-product depends upon culture or geographical region. A large international trade exists for these products due to their low cost and high nutritional value. However, these edible by-products are mainly limited to organs such as brain, heart, kidney, liver and tripe due to palatability, customs, religion and reputation (Ockerman et al., 2017). Offal from lamb, beef and pork are popular and prepared in different ways (Table 2.1).

2.1.1 Consumer preference

Beliefs and attitudes affect the consumer's perception of various products. Acceptance or rejection of a product is mainly determined by beliefs as it could affect the image as well as the perception of the product. Consumers' choice of products, selection, and interpretation of information for immediate decision making is defined as consumers' perception. At the point of purchase, consumers form their feelings about the quality expectations for a meat product. Norms, customs and traditions of a particular society could affect the acceptability of meat offal in that society. Therefore, assessing the quality of offal could be influenced by individual preferences related to cultures and societies (Alao et al., 2018).

Table 2.1 Preparations of different offal

Offal type	Means of storage and preparation	Usage method
Lamb		
Liver	Frozen, fresh, refrigerate Grind, sliced or whole	Sausage, fry, patty, loaf, broiled, braised
Kidney	Fresh or refrigerate Whole or sliced	Broiled, cooked in liquids, braised, stew, fried, soup
Heart	Frozen, fresh, refrigerate Grind, sliced or whole	Braised, cooked in liquid, luncheon meat, roasted, loaf, patty, sausage ingredient, stuff
Stomach	Fresh or refrigerate	Honeycomb tripe
Testicles	Fresh, frozen, refrigerate	Fried
Beef & veal		
Liver	Frozen, fresh, refrigerate Grind, sliced or whole	Sausage, fry, patty, loaf, broiled, braised
Kidney	Fresh or refrigerate Whole or sliced	Broiled, cooked in liquids, braised
Heart	Fresh, frozen, refrigerate Whole or sliced	Braised, cooked in liquid
Tripe	Fresh or refrigerate Pre-cooked, soak or pickled before use	Fry, boiled, cooked in liquid
Pork		
Liver	Frozen, fresh, refrigerate Grind, sliced or whole	Sausage, fry, patty, loaf, broiled, braised
Kidney	Fresh or refrigerate Whole or sliced	Broiled, cooked in liquids, braised, grill, stew and soup
Heart	Fresh, frozen, refrigerate Whole or sliced	Braised, cooked in liquid, loaf, patty, luncheon meat, sausage ingredient
Stomach	Fresh, refrigerate, pre-cooked	Broiled, cooked in liquid, sausage ingredient

Adapted from Hui, Y. H., Nip, W.-K., & Rogers, R. (2001). *Meat science and applications*: CRC Press.

Though a high demand for meat consumption prevails, the demand for meat co-products has decreased globally (Lynch et al., 2018). Offal may be rejected by some individuals due to disgust which is associated with the nature and origin of the food. Individuals who have never eaten offal before have associated offal consumption with the consumption of living organs (Henchion et al., 2016), which resulted in rejection of offal. An offal that had been consumed during childhood was not questioned for its appropriateness as a food, whereas, an offal product that was not consumed in childhood was. The lack of information related to the health benefits of offal consumption was also put forward as a reason for the negative perception towards offal consumption (Henchion et al., 2016).

Despite offal having a higher nutritional value it maintains preferential consumption with some consumers who are fond of eating it and others who are not. Though prominent product parameters such as nutritional, health and sensory properties are important, the purchasing of organ meats is mainly affected by social, cultural and economic parameters to a great extent (Ayroe et al., 2016; Ojewola and Onwuka, 2001). Offal marbling and packaging, taste and consumer preference are some factors that affect the purchasing of offal (Ayroe et al., 2016; Langyintuo et al., 2004). In a study conducted in Kumasi metropolis with high consumption of cattle and goat offal, preference for stomach and liver were found to be 52.70% and 38.10% respectively. (Ayroe et al., 2016). Among the reasons for preference for offal, nutritional value (58%) was the most dominant reason followed by delicacy (40%) while the cost (2%) was of lowest concern. The educational background of the respondents seemed to have a positive influence on preferential consumption of meat co-products as majority of the respondents were elite (48.5%) (Ayroe et al., 2016). In a separate study conducted in Kumasi metropolis, to evaluate the preferential consumption of pig offal, the results revealed that the liver (32%) was the most preferred organ whereas the heart (3%) was the least preferred (Nonterah et al., 2015). In this study, it was found that offal from the open market was preferred more than those from the supermarket and butcher's shops. This may be due to the varieties, affordability and accessibility of offal in the open market. The occupation of the respondents positively influenced the higher preference for liver as majority of the respondents were well aware of the nutritional value of the liver (Nonterah et al., 2015). In a study conducted in Amathole District of South Africa, to study the factors determining consumer preference towards the price of sheep and cattle offal (Alao et al., 2018), it was revealed that the highly preferred offal was liver (94.1%) followed by tripe (78.2%) and intestine (68.8%). Among the available retail outlets, the consumers preferred butcheries over supermarkets mainly due to the availability of fresh and cheap offal which was readily accessible. The price of offal also was a major factor that affected the purchasing decision. The preference toward products sold in the butchery was

mainly due to the larger offal quantity offered for a cheaper price (Alao et al., 2018). In a study where the meat consumption behavior was explored in Jammu district, India, the results revealed that a high proportion of the respondents (51.70%) consumed offal and 35% among them believed offal is healthy (Ali et al., 2017). Red offal (liver, kidney and heart) was preferred more followed by dark offal (head and feet) and grey offal (stomach, intestine, lungs and spleen). Results further revealed that purchasing meat from the clean retail meat shop (55%) and affordability (65%) was preferred the most, which highlights that the cleanliness and freshness as well as decent pricing of the co-products was highly preferred at the point of purchasing (Ali et al., 2017).

2.1.2 Offal consumption around the world

Though the global demand for offal meat has declined and revulsions may be exhibited towards offal, consumption of certain offal dishes are considered as delicacies in various parts of the world. In Brazil, chicken hearts are consumed grilled or roasted and in Peru bovine hearts are served as a part of traditional gastronomy. In Japan, Russia, Mediterranean countries and in some South American countries, beef tongue is considered as a delicacy. Pork, beef and lamb kidneys are much appreciated in the United Kingdom. Traditional dishes from Scotland, Rumania, Turkey, Bulgaria and Spain include beef tripe and use the porcine tripe as a casing for dry and semi dry fermented products. Tripe is also consumed in countries such as Mexico and Cuba as a "mondongo" preparation. China consumes beef tripe as a cold appetizer (Nollet et al., 2011; Lynch et al., 2018). In Africa, almost all of the edible co-products are processed and consumed as traditional dishes (Alao et al., 2017). Organ meat from bovine, duck, pork and chicken are generally used in soup preparations in China, Korea and Singapore. These organ meats may also be consumed by dipping in soy sauce. Goat offal such as feet, head, testicles or tongue is considered a delicacy in India, Pakistan, Nepal and Bangladesh. Iran serves kebabs made of sheep's liver, heart and kidney (Nollet et al., 2011; Lynch et al., 2018).

2.1.3 Foodborne diseases associated with offal consumption

In certain countries offal are consumed raw. For instance, Koreans consume raw beef tripe and liver which are served as a side dish (Jeong et al., 2017). A study conducted in South Korea predicted that for home consumption, the *Campylobacter* foodborne illness probability per person per month for home cooked food and restaurant cooked food are 1.56 3×10^{-5} and 1.74 3×10^{-5}, respectively. These results clearly indicate risk of *Campylobacter* foodborne illness due to the consumption of raw beef offal (Jeong et al., 2017). Chicken liver is a commonly known vehicle for campylobacteriosis and salmonellosis. Many outbreaks due to the

consumption of chicken liver had been reported in previous studies (Glashower et al., 2017; Little et al., 2010; Merritt et al., 2011). Inadequate cooking and pathogen contamination were known as the major reasons for these outbreaks (Lanier et al., 2018). In New South Wales (Australia), cases of salmonellosis were linked to the consumption of contaminated lamb's liver or food cross-contaminated with lamb liver during preparation (Hess et al., 2008). Previous other studies have also revealed an association between offal and salmonellosis due to food handling error and cross contamination (Layton et al., 1997; Cornell et al., 1998). The raw consumption of offal as well as its association with major foodborne diseases emphasise the need to investigate the microbiological quality of edible offal.

2.2 Foodborne Diseases

Foodborne diseases are a major issue in developed as well as developing countries. Each year over 100 million people are exposed to foodborne and waterborne diseases worldwide (Dallal, 2014) with the consumption of food contaminated with pathogenic bacteria being the main cause for this hazard. Epidemiological studies have revealed that foodborne diseases are mainly caused by food of animal origin (Dallal, 2014). Food is exposed to contamination at many food handling stages such as production, processing, distribution and retail (Dallal, 2014). Living animals carry pathogenic bacteria and they are harboured within the processing environment. Pathogenic bacteria could contaminate meat products during the slaughter process where the carcass would be subjected to contamination. The safety of meat products could be ensured by implementing measures to minimize contamination and inactivating pathogens (Borch & Arinder, 2002).

In New Zealand, salmonellosis is the second major cause of notified bacterial human enteritis with rates of 28.90 and 37.00 per 100,000 population in 2004 and 2005, respectively (Wong et al., 2007). In New Zealand, salmonellosis outbreaks due to *Salmonella brandenburg* have been linked to endemic disease in sheep.

The National Foodborne Diseases Surveillance Network in China reported in the foodborne disease outbreak report (1992-2005) that salmonellosis was the second major cause of foodborne diseases of bacterial origin. Furthermore, 10-20% of outbreaks related to *Salmonella* were reported to occur annually (Yan et al., 2010).

Based on the incidence data collected from 45 countries, listeriosis has been found to lead to 5463 global death in 2010 (de Noordhout et al., 2014). The Chinese National Centre for Food Safety Risk Assessment (CFSA) has reported a total of 147 food-borne listeriosis cases in the

period from 1964-2010 (Zhou et al., 2017). Meat is considered as the major source of *L. monocytogenes* infections (Li et al., 2018; Gallagher et al., 2003). Ready-to-eat (RTE) meat gained special attention in this regard as it is consumed without being subjected to further decontamination or processing hurdles (EFSA, 2018; WHO, 2004). In New Zealand, listeriosis is considered to be a rare disease with an approximate rate of 0.40 per 100000 population (21 cases reported in 2017) (Rivas et al., 2019). A study performed in New Zealand in 2013 had reported that about 86.00% listeriosis was foodborne and from this 54.10% was due to processed RTE meats (Rivas et al., 2019).

Campylobacteriosis is considered as one of the most frequent infectious gastrointestinal illnesses occurring in humans worldwide (Butzler, 2004). In New Zealand, it is considered as the most reported notifiable disease (Anderson et al., 2012). *Campylobacter jejuni* and *Campylobacter coli* are the most common pathogens held responsible for this disease in New Zealand and worldwide, being responsible for about 80–85% and 10–15% of cases, respectively. The disease is mainly foodborne specially through poultry, but may be caused by other animals as well (Bojanić et al., 2017). In Northern China, 36 cases of Guillain–Barre syndrome triggered by *C. jejuni* were detected in 2007 (Zhang et al., 2016). It was reported in a study conducted from 2008 to 2014 that *C. jejuni* and *C. coli* were recovered at rates of 18.1% and 19% respectively in chicken obtained from Chinese provinces. (Y. Wang et al., 2016).

Table 2.2 Incidence of foodborne pathogens in edible meat offal

Microorganisms	Processing conditions	Location	Animal	Prevalence of microorganism										Reference
				H	**L**	**Lu**	**T**	**SI**	**LI**	**LUM**	**O**	**B**	**K**	
***Salmonella* spp.**	Samples washed, trimmed and chilled at 4^0C	Slaughterhouse, Korea	Pig	3/11	2/11	2/10	1/10	4/10	3/11	-	-	-	-	(Im et al., 2016)
			Cattle	0/6	0/6	0/6	3/13	0/6	0/5	-	-	-	-	
	Samples placed at 4^0C immediately transferred for analysis	Abattoirs (A) and Butcheries (B) in Afyorikarahisar, Turkey	Cattle	-	7/60	-	-	-	-	-	-	2/25	4/60	(Akkaya et al., 2012)
	Washed raw samples	Slaughterhouses in Gyeongsangbuk-do province, Korea	Pig	1/9	0/9	1/9	0/9	0/9	1/9	-	-	-	-	(Lee & Lee, 2016)
			Cattle	0/8	0/8	1/8	-	1/8	0/8	1/8	1/8	-	-	
	Abbreviations: Heart (H), liver (L), lung (Lu), tripe (T), small intestine (SI), large intestine (LI),lumen (LUM), omasum (O) brain (B), kidney (K)													
	Fresh raw samples collected at point of sale	Retail, food service premises, UK	Cattle	3/49 (liver, heart, kidney, oxtail, tripe)										(Little et al., 2008)
			Sheep	5/161 (liver, heart, kidney),										
			Pig	31/131 (liver, heart, kidney, tripe)										
***Campylobacter* spp.**	Raw samples	Abattoirs (A) and retail butchers (B) in 5 Lancashire towns		A		B		Liver (mostly), kidney, heart						(Bolton et al., 1985)
			Cattle	9/56		7/97								
			Pig	41/107		30/125								
			Sheep	1/41		3/26								
	Raw samples	Butcher's shops (B) and supermarkets (S) New Zealand in autumn (A) and spring (SP)	Sheep	B	S	A	SP	Livers						(Cornelius et al., 2005)
				83/140	97/132	101/136	79/136							
		Retail, food service premises, UK	Cattle	6/49 (liver, heart, kidney, oxtail, tripe)										(Little et al., 2008)

	Fresh raw samples collected at point of sale											
			Sheep	59/161 (liver, heart, kidney),								
			Pig	24/131 (liver, heart, kidney, tripe)								
	Retail supermarkets and butcher's shops Aberdeen, NE Scotland	Raw samples	Chicken	21/26				Liver				(Strachan et al., 2012)
			Cattle	22/32								
			Pig	23/29								
			Sheep	31/40								
Listeria monocytogenes	Raw samples	Three wet markets (WM) in Selangor	Cattle		WM 1			WM 2		WM 3		(Kuan et al., 2013)
				Liver	0/5			3/5		1/6		
				Lung	¼			¾		4/6		
				Intestine	2/4			0/5		-		
				Tripe	2/5			3/5		2/5		
				Spleen	0/4			0/5		-		
	Raw samples	Poultry farms and markets in El-Gharbia governorate, Egypt	Chicken	Liver				21/100				(Abd El-Tawab et al., 2018)
				Spleen				8/100				
				Kidney				3/100				
	Raw samples	Four retail wet markets (WM) and three hypermarkets (HM) in Selangor, Malaysia	Chicken		WM1	WM2	WM3	WM4	HM1	HM2	HM3	(Kuan et al., 2013)
				Liver	2/10	4/9	0/7	5/10	3/12	3/12	1/12	
				Heart	11/61	2/13	0/8	0/9	4/12	3/12	5/12	
				gizzard	1/4	1/8	3/10	6/14	6/12	3/12	4/12	
Clostridium perfringens	Samples washed, trimmed and chilled at 4^0C	Slaughterhouse, Korea		H	L	Lu	T	SI	LI	LUM	O	(Im et al., 2016)
			Pig	0/11	0/11	0/10	0/10	4/10	3/11	-	-	
			Cattle	0/6	0/6	0/6	2/13	1/6	0/5	-	-	
	Washed raw samples	Slaughterhouses in Gyeongsangbuk-do province, Korea	Pig	0/9	0/9	0/10	0/9	2/9	0/9	-	-	(Lee & Lee, 2016)
			Cattle	0/8	0/8	0/8	-	1/8	0/8	0/8	1/8	

2.2.1 Enterobacteriaceae

Bacteria belonging to *Enterobacteriaceae* family are gram-negative, non-spore forming and are found in the intestinal tracts of animals (Manhique et al., 2020). *Hafnia alvei, Serratia* spp. and *Enterobacter* spp. are some such species that have been found to cause food spoilage when present in high numbers (>10^7 CFU/g). As some members of the *Enterobacteriaceae* family (non-typhoidal *Salmonella* and Shiga toxin-producing *E. coli)* (Colavecchio et al., 2017) are highly pathogenic, and high counts indicate the possibility of food poisoning and infections, these bacteria are used to evaluate the hygienic quality of food as they are present in faeces of warm-blooded animals and it is easy to detect *Enterobacteriaceae* as indicators of sanitation than coliforms (group within the *Enterobacteriaceae* family) as they exhibit greater resistance towards the environment than the latter (Manhique et al., 2020). The presence of coliforms (consist of 10% of intestinal microbiota) in food also indicate poor hygienic practices in food handling. It is also a useful indicator to evaluate the effectiveness of microbial control measures and the safety of food (raw or processed). Coliforms and *E. coli* counts are indication of faecal contamination in food. However, the International Commission on Microbiological Specifications for Foods (ICMSF) has not define a specific criteria for *E. coli* and coliforms counts to be used as an indicator of enteric bacterial contamination of edible offal (Im et al., 2016). However, individual countries may have adopted their own standards, For example, standards are set by the United Kingdom for *E. coli* count it should not exceed 4 log CFU/g in processed products (Im et al., 2016).

In a study conducted by Cohen et al. (2006) it was reported that the faecal coliforms present in chilled, fresh samples of beef, lamb and beef offal (heart and liver) (collected from butcheries, supermarkets and slaughterhouses) were 2.50, 2.80 and 2.30 log CFU/g respectively. The study also revealed that the average coliform numbers isolated from the samples obtained from butcheries and supermarkets were found to be significantly higher than those collected from the slaughterhouses. In a cattle slaughterhouse, the workers' hands and knives showed a significant reduction in total viable counts when treated with hot tap water (82^0C) in between various stages of processing (Abdalla et al., 2010). This indicates that if not properly sterilized meat equipment, as well as the workers, could contribute towards cross contamination. The higher incidence of coliforms in samples from butcheries and supermarkets may have been due to this.

In a study conducted by Im et al., (2016), 41.40% of pig green offal, which is intestinal contents (stomach, small intestine, large intestine), 3.10% of pig red offal (heart, liver, lung) and 11.80% of cattle green offal were found to exceed 4.00 log CFU/g of *E. coli*. The higher incidence of *E. coli* in green offal is justified due to the natural occurrence of intestinal microflora. However,

the higher counts observed in green offal which were processed (washed, trimmed and chilled at 4^0C) only indicates that inefficient meat decontamination procedures were carried out. Effective washing is had been found reduce residual contamination in ruminal contents in a previous study (Bensink et al., 2002). In Australia, wet dumping is carried out which is a process where 20-30 L of water is used to empty each paunch. In a study where paunches were emptied using 30 L of water and rumen pillars (trimmed and washed) and tripe (scalded with water at 80^0C) were frozen 95% total coliform count values for both products were found to be less than 4 log/cm^2 (Bensink et al., 2002).

Ibrahim et al. (2013) reported the *Enterobacteriaceae* counts of chilled cattle liver, kidney and lung obtained from slaughterhouses in Egypt to be $84\times 10^3 \pm 18\times10^3$, $69\times10^3 \pm 17\times10^3$ and $84\times10^3 \pm 21\times10^3$ CFU/g, respectively. According to the European Commission (2007) standards, these values are higher than the permissible level of *Enterobacteriaceae* in edible offal which is 3.17×10^2 CFU/g. In the same study coliform counts of cattle liver, kidney and lung were found to be $34\times 10^3 \pm 7\times10^3$, $22\times10^3 \pm 5\times10^3$ and $32\times10^3 \pm 8\times10^3$ CFU/g, respectively. Total coliform counts are an indication of the sanitary conditions in the food processing environment. Much lower *Enterobacteriaceae* counts were reported by Abdullah et al. (2008) for sheep liver, kidney, spleen and heart were less than 10^3 CFU/g. These are samples were also from a slaughterhouse in Jordan which were cut with a sterile knife, washed and stored at -18^0C till analysis was performed. These findings indicate that if proper sterile conditions are maintained during the slaughtering process till storage better microbiological quality could be ensured for meat.

However, *E. coli* O157:H7 was not isolated from any of the offal samples. A similar finding was reported by Lee & Lee (2016) where sheep and cattle offal were studied. *E. coli* O157:H7 is a common foodborne pathogen isolated from red meat and due to its high pathogenicity in humans its occurrence is heavily investigated.

2.2.2 Aerobic plate counts (APC)

The general indicator of microbial contamination is measured by APC. When APC of the meat exceeds 7 log CFU/g meat is considered to be unfit for human consumption according to the ICMSF criteria. However, the criteria also states that out of five samples more than three samples should have an APC <6 log CFU/g for offal to be approved to have satisfactory hygienic quality (Im et al.,2016). Cohen et al. (2006) reported that the mean APC counts obtained for beef, lamb and beef liver (fresh and chilled) were 6.5 l, 6.2 l, and 5.0l log CFU/g

respectively. Cohen et al. (2006) used samples from slaughterhouses, butcheries and supermarkets in Morocco for his study and he observed an increase in the APC of the supermarket samples compared to samples from other two locations. Poor hygienic conditions and meat being stored at inadequate temperature during transport and storage may have resulted in this. Im et al., (2016) reported that APC counts in pig and cattle red (heart, liver, and lung) and green offal (stomach, small intestine, and large intestine) purchased from a slaughterhouse all of the samples had APC values which agreed ICMSF criteria (< 7 log CFU/g). However, Hannah et al. (1982) reported that beef, pork and lamb offal (livers, kidneys and hearts) soon after slaughter carry microbial flora of nearly < 10^4 or <10^3 log CFU/cm^2. Furthermore, the study revealed that up to five days of storage at 2^0C did not significantly affect the APC in offal, but after day five a significant increase could occur. It was also observed that when the organs were subjected to temperature abuse (6-12 hours at 30^0C) prior to freezing major increases in APC were observed (Hanna et al., 1982). Lower APC counts have also been reported by Abdullah (2008) where he reported APs ranging from 2.25 to 4.45 in the liver, spleen, kidney and heart (frozen) of Jordanian sheep from a local slaughterhouse.

Chilled pork offal samples (heart, kidney, brain, liver and intestine) collected from pork processing plants revealed that only intestines had samples (14.7%) which exceeded the APC of 7 log CFU/g being unfit for consumption according to ICMSF criteria. The rest of the offal samples had APC counts, which averaged around less than 5 log CFU/g. The sampling was conducted within 96 hours of samples collection (Erickson et al., 2019). These findings indicate that disinfection procedures for intestines need to be thoroughly monitored as they are more prone to bacterial incidence due to the natural inheritance of gut microflora.

2.2.3 *Listeria monocytogenes*

Listeria are ubiquitous bacteria widely present in the environment and contaminate food products inevitably. It is a psychrotrophic organism capable of surviving and growing at chill temperatures (Wai et al., 2019). Epidemiological studies have shown that *Listeria monocytogenes* (*L. monocytogenes*) is a significant food-borne pathogen. *L. monocytogenes* is a halotolerant microbe capable of surviving in low pH and high salt concentrations. *L. monocytogenes* prevalence of 45% has been reported in Mediterranean-style dry fermented sausages in a previous study (Meloni, 2015). Dairy products as well as food of animal and vegetable origins are sources of listeriosis outbreaks. *Listeria* spp. has been isolated from meat and meat products around the world and it is regarded as a safety concern since the bacteria can thrive in cooled meat at refrigeration temperatures. Determining the *L. monocytogenes* prevalence in cooked meat is rather important as exposure to heat for brief periods would be

insufficient to destroy all viable cells. Listeriosis is identified as a major public health issue worldwide as up to 30% of cases are fatal (Yücel et al., 2005).

In a study where Kuan et al. (2013) investigated the presence of *L. monoycytogenes* in beef offal collected from different wet markets the overall (combining results from all three wet markets) highest incidence of *L. monocytogenes* was in beef lung (50%) followed by the tripe (46.67%), liver (25.00%) and intestine (22.22%) (Table 2.2). There were none present in the analysed spleens. Different wet markets showed different levels of *L. monocytogenes* contamination, which is likely to be a result of different handling practices carried out at the different locations. The use of unhygienic containers for transportation and distribution, cross contamination via other contaminated food, improper practices of food handling are major modes which contamination could occur. This is clearly evident in the study conducted by (Kanarat et al., 2011) where they reported that chicken at the initial stage of production had no *L. monocytogenes* but the frozen chicken meat and the RTE chicken meats were found to have *L. monocytogenes* in 2.50% and 0.20% samples, respectively. These results indicate improper hygienic practices carried out at the slaughterhouse.

Storage temperature also plays a vital role controlling the distribution of food pathogens. The control of the initial *L. monocytogenes* load on beef offal is limited when stored under refrigeration temperatures. This is mainly due to their psychrotrophic nature, which enable them to thrive and proliferate in cold environment. Therefore, the longer the beef offal is held at such temperatures, the higher will be its microbial load (Kuan et al., 2013). A study that investigated poultry offal in Egypt revealed that a higher incidence of *L. monocytogenes* in liver (5.25%), compared to the kidneys (3.25%) and spleen (2.00%) (Abd El-Tawab et al., 2018) (Table 2.2). However, in a different study by Kuan et al. (2013) reported a much higher incidence of *L. monocytogenes* in chicken offal (gizzard was 33.33%, liver was 25.00% and heart was 20.83%). These results were still much lower than the incidence observed by Arumugaswamy et al., (1994) in chicken liver and gizzard that showed the presence of 60% and 66% *L. monocytogenes* respectively. Cohen et al. (2006) reported that *Listeria* were not detected in cattle offal samples as well as in samples of sheep meat and cattle meat collected from a butchery, supermarket and slaughterhouse (transported to the laboratory at 4^0C) in Morocco. Since *L monocytogenes* is a psychrotrophic pathogen it is capable of thriving and proliferating in temperatures below 4^0C this confirms that meat samples were free of *L. monocytogenes* at the point of sale. Therefore, this is due to the effective implementation of hygiene control, good manufacturing practices or hazard analysis and critical control points (HACCP). Practice of these measure ensure that transmission and cross contamination caused by food pathogens is minimized (Kuan et al., 2013). In a Iranian study conducted by (Mashak et al., 2015) including different meat (chicken,

turkey, quail, beef, sheep, camel and ostrich) except for chicken the contamination in fresh meat was equal or greater than frozen meat. In the study *the L. monocytogenes* positive samples in beef meat (fresh, fresh minced and frozen) was significantly higher than in other meat. Furthermore, there was no *L. monocytogenes* detected in the frozen sheep meat samples (n=24). Inaccuracies in the food processing plants have been found to be the major cause for the Listeria outbreaks (Todd & Notermans, 2011). Contamination has also been found to occur via contaminated equipment and personnel (Gudbjörnsdottir et al., 2004).

When investigating the occurrence of four cases of listeriosis in a New Zealand hospital it was revealed that that *L. monocytogenes* isolates detected in RTE meat (from a RTE producer) provided to the hospital matched with two pulsotypes identified from the clinical isolates. Furthermore, RTE meats from the hospital kitchen were also found positive for *L. monocytogenes* (Rivas et al., 2017). In a New Zealand study where packaged RTE meats (n=1485) from 32 New Zealand producers were investigated for the presence of *L. monocytogenes* the prevalence in the survey was found to be 6.4% *L. monocytogenes* (Rivas et al., 2017). Much lower prevalence was reported in previous New Zealand studies. In 2005, *L. monocytogenes* prevalence in unpackaged ham was found to be 4.3% (Cornelius et al., 2008). and from 2003-2004 a prevalence of 1% was reported in vacuum packaged ham (Wong et al., 2005). In most cases when obtaining a second sample from a premise which provided *L. monocytogenes* positive sample the second sample was found negative for the pathogen. This indicates the contamination is sporadic rather than persistent contamination (Cornelius et al., 2008). In a United States study where the microbiological quality of imported boneless beef trim meant for ground beef was investigated it was found that out of 219 samples imported from New Zealand, six samples were positive for *Listeria* spp. (Bosilevac et al., 2007).This indicates the possibility for *Listeria* prevalence in imported ruminant meat. However, there are no results published for the *Listeria* prevalence in sheep offal imported by New Zealand. Therefore, the present study focusses on investigating the presence of *Listeria* in New Zealand sheep offal.

In a meta-analysis conducted in China including publication published from 2007-2017 the pooled prevalence of *L. monocytogenes* in fresh pork, fresh beef, fresh poultry, frozen raw meats, fresh mutton and RTE meats were found to be 11.30%, 9.10%, 7.20%, 7.20% ,5.40% and 3.20% respectively. The study showed a higher *L. monocytogenes* incidence in raw meat compared to RTE meats (Liu et al., 2020). A percentage of 59.20% of chilled pork samples have been found positive for *Listeria* spp. in a study conducted in China. A higher incidence of *L. innocua* (91.83%) was reported followed by *L. monocytogenes* (19.39%) and *L. welshimeri* (7.14%) (Fang et al., 2016). These findings clearly indicate the possibility of occurrence *Listeria* spp. in red meat.

L. monocytogenes has been found to be present in environment and equipment in a food processing plant even after disinfection (Wendlandt and Bergann, 1994). *L. monocytogenes* is capable of replicating within a slaughterhouse, which has been subjected to contamination at least once. Cross contamination could occur between the hand of personnel and carcass spreading the pathogens. For this reason *L monocytogenes* eradication is known to be difficult and slaughterhouses are known as primary sources of carcass contamination (Wendlandt & Bergann, 1994).

2.2.4 *Clostridium perfringens*

Clostridium perfringens (*C. perfringens*) is a non-motile, bacillus and is grouped into five toxin types, (A, B, C, D, and E) based upon the four major toxins (alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX)) produced by them. Human gastroenteritis is caused by types A and C that can grow in temperatures ranging from 15 - 50^0C. Moreover, the generation time is found to be less than 20 minutes for temperatures between 33-49^0C (Tizhe et al., 2015). Strains are capable of producing heat sensitive as well as heat resistant spores. Some spores could be killed at 100^0C in few minutes but some would survive boiling for 1-6 hours. With regard to nutritional needs, *C. perfringens* is quite demanding. For this reason, meat products are highly prone to *C. perfringens* contamination as they are capable of meeting the pathogen's amino acid and vitamin requirements (Bryan, 1969). Among pathogenic bacteria, *C. perfringens* is widespread in the environment and present in healthy animals within the gastrointestinal tract. The slaughtering process is the major source of contamination. Sulfite-reducing clostridia are known to be the most fatal of anaerobic microbes responsible for food spoilage. These organisms may grow within the carcass and result in the production of toxins and off flavours (Cohen et al., 2006).

Im et al., (2016) reported that pig offal samples were tested microbiologically and the third most common pathogen isolated from the samples was *C. perfringens* (11.10%) that dominated the small and large intestines. In the same study, tested cattle offal were found to have *C. perfringens* (7.10%) as most dominant pathogen in the stomach and small intestine alongside *Salmonella* (Table 2.2). A much lower *C. perfringens* occurrence was reported by Lee & Lee (2016) where *C. perfringens* was only isolated from two pig small intestine samples (3.70%) and one cattle omasum (1.80%) (Table 2.2). In both studies, since gut related offal showed presence of *C. perfringens*, it could be deduced that the decontamination procedures for gut related offal (small intestine, large intestine and omasum) need to be well monitored, as they are natural reservoirs of the pathogen.

In a study *C. perfringens* was found to be present in samples from trucks and lairage samples obtained after cleaning and disinfection in a pig abattoir (Álvarez-Pérez et al., 2018).This finding indicates the disinfection procedures were insufficient to eliminate the clostridial spores. Findings also suggest the need to implement exhaustive cleaning and disinfection protocols at each step from transportation of animals to lairage.

When 400 raw beef samples purchased from seven markets in Nigeria were examined for the presence of *C. perfringens* only 3.05% of samples were found to be positive for the pathogen (Tizhe et al., 2015). However, the low incidence in the samples were assumed to be a result of the heat shock treatment (10-15 minutes at 80^0C) the samples underwent to eradicate non-spore forming aerobic bacteria. Findings reported by Qiyi and McClane (2004) confirm this as they found *C. perfringens* to be present in heat chocked and non-heat shocked samples at rates of 2.00% and 29.00% respectively.

In a study performed in India 68.80% of healthy sheep and 55.00% of healthy goats were found to be positive for *C. perfringens*. Furthermore, 69.77% of isolates obtained from healthy sheep were positive for toxinotype A while 30.23% were positive for toxinotype B (Nazki et al., 2017). These findings in healthy sheep indicate that a possibility for sheep offal contamination during meat processing. Therefore, the incidence of *C. perfringens* in sheep offal will be investigated in the present study.

2.2.5 *Salmonella* spp.

Salmonella is a zoonotic agent that could survive within the intestines of healthy animals showing no symptoms and passed on to the environment via faeces. Faeces as well as the transfer of organisms on animal's hides could result in contamination of holding and stunning areas. *Salmonella* is capable of surviving in the environment for long periods of time transferring into the skins and hides of animals handled within the same facility (Small et al., 2006). Salmonellosis is the most common foodborne illness after Campylobacter infection and the major cause of foodborne infections. Consumption of contaminated food such as milk, seafood, egg and red meat leads to the infection in humans. Until distributed in the markets the pathogen can survive within food products especially in meat (Dallal, 2014).

Im et al., (2016) reported *Salmonella* was the most frequently isolated food pathogen among several others from pig (23.80%) and cattle (7.10%) offal (Table 2.2). However, Little et al. (2008) observed a higher prevalence of *Campylobacter* in lamb and beef offal (purchased from retail supermarkets and butcher shops) in comparison to the *Salmonella* occurrence. Among the different types of offal, *Salmonella* was mostly prevalent in pork offal (23.60%) and less prevalent in beef (6.10%) and lamb (3.10%) (Table 2.2). According to Lee & Lee (2016) a

higher proportion of *Salmonella* were detected in cattle offal (7.14%) than in pig offal (5.55%) (Table 2.2). These differences could be due to reasons such as the impact of feed withdrawal and transportation. Feed withdrawal is the total time period during which animals are deprived of food during transport and waiting time at the slaughterhouse prior to slaughter. Inadequate feed withdrawal times would lead to intestines partially filled with feed and faeces. This could lead to carcass contamination via ruptured intestines. When animals are being transported if overcrowding occurs this could stress the animals leading to increased peristaltic movement sin the intestines leading to excretion of faeces and pathogens (Rasschaert et al., 2020).

Contamination generally occurs during the process of slaughter and evisceration (Little et al., 2008). Also in countries such as Korea, offal are contaminated due to improper handling in the absence of approved regulations (Lee & Lee, 2016). Akkaya et al. (2012) reported that the amount of *Salmonella* present in cattle offal samples from abattoirs were slightly higher than in the samples from the local butcheries. This reveals the presence of multiple cross contaminants during post slaughter processing and handling of offal and the premise's hygienic standards. The lower *Salmonella* incidence in the local butcheries may also due to the better treatment of material to represent high value in contrast to companies that focus more on the meat. Cohen et al. (2006) reported that Salmonella was not detected in cattle offal obtained from a slaughterhouse, butchery and supermarket in Morocco. This reveals that proper practices of handling and personal hygiene could minimize the contamination of meat.

In a New Zealand study which was conducted from 2003-2005 to investigate the prevalence of *Salmonella* in uncooked retail meat it was observed that in chilled samples of chicken, lamb, un-weaned veal, beef and pork 7, 3, 1, 1 and 0% of *Salmonella* was present (Wong et al., 2007). Another study from New Zealand where RTE meat samples from 32 New Zealand producers were investigated revealed the absence of *Salmonella* in all samples (Rivas et al., 2017). In a previous study (Wong et al., 2009) had reported the absence of *Salmonella* in pig carcasses collected from two pork processing plants. In New Zealand the pork processing operates under HACCP based Risk Management Programmes. The results of the study indicate the efficiency of the programme (Wong et al., 2009).

A Chinese study where meat samples, obtained from open-air markets and large supermarkets, were analysed for the presence of *Salmonella* results revealed that retail meat, pork, chicken, beef and mutton had 23.9, 26.7, 15.8, 33.3 and 33.3% *Salmonella* incidence (Yan et al., 2010). In another Chinese study from Shanghai Xu et al., (2018) reported that *Salmonella* was isolated from farm products obtained from supermarket, farmers' markets, slaughterhouse and dairy farms were investigated, pork, chicken, beef and mutton showed *Salmonella* positive rates of 42.40, 41.20, 31.30 and 16.70%, respectively. Moreover, meat (37.50%) showed the highest

Salmonella prevalence compared to vegetables (4.16%), milk (0.00%) and raw eggs (0.00%) (Xu et al., 2018). A similar trend was observed in another study from Shanghai. Prevalence of *Salmonella* in pork, chicken, beef, duck and mutton were found to be 32.20, 27.50, 24.30, 24.00 and 20.00% respectively (Yang et al., 2019). These results clearly indicate that meat is a major source of *Salmonella* while depicting the need to investigate *Salmonella* incidence in red meat.

2.2.6 *Campylobacter* spp.

The infection is commonly found to be caused by raw or undercooked poultry or by-products, which are exposed to contamination. Studies on poultry has showed that *C. jejuni* was dominant over *C. coli*. However, the ratio among these two strains were found vary depending on the country (Suzuki & Yamamoto, 2009). Studies conducted in many countries have indicated that 2 - 14% of patients suffering from acute gastroenteritis are infected by *C. jejuni* at a similar level to *Salmonella* (Kwiatek et al., 1990). *Campylobacter* spp. are generally present in the intestinal tracts of pig and cattle. In spite of this, *Campylobacter* spp. has not yet been strictly associated with red meat consumption (Borch & Arinder, 2002). Studies show red meat offal as a *Campylobacter* reservoir (Little et al., 2008; Strachan et al., 2012). Despite red meat offal could potentially be a health hazard for humans and there are limited studies on (Bolton et al., 1985; Little et al., 2008; Strachan et al., 2012) *Campylobacter* incidence it is important to further investigate these meat types.

Bolton et al. (1985) reported the presence of *Campylobacter*, in offal samples (mostly liver with less amounts of kidney and heart) of cattle, sheep and pig obtained from retail butchers and abattoirs (Table 2.2). The authors mostly found *Campylobacter* in sheep offal (6.00 – 30.00%) followed by cattle (5.00 – 10.00%) and pig offal (6.00%). A somewhat similar finding was reported by Little et al. (2008) where 36.66% of sheep offal (liver, heart and kidney) were *Campylobacter* positive and less contamination was present in pork offal (liver, heart, kidney and tripe) (18.30%) and beef offal (liver, heart, kidney, tripe and oxtail) (12.24%) (Table 2.2).The difference maybe a result of the intestinal carriage of *Campylobacter* in each animal. The study further revealed that more *Campylobacter* was isolated from abattoirs than butcher shops. This was similar to the finding by Cornelius et al. (2005) where a high prevalence of *Campylobacter* was observed in supermarket bought sheep liver (73.48%) in comparison to those purchased from the butcher's shop (59.28%) (Table 2.2). This may be due to the high atmospheric oxygen contents the meat samples are exposed to in retail environments which is unfavourable for microaerophilic organisms (Bolton et al., 1985). Strachan et al. (2012) revealed the presence of high incidence of *Campylobacter* in livers of chicken (81.00%), pig (80%), cattle (69.00%) and sheep (77.5%) (Table 2.2). The study further revealed the isolates

of Campylobacter spp. obtained from chicken (56%) showed belonged to the top 10 human genotypes the most followed by cattle (20%), pig (18%) and sheep (13%).

The positive *Campylobacter* incidence in the livers obtained from chicken, cattle, pig and sheep studied indicates the risk posed by liver consumption. Proper handling of the food alongside cooking the livers well could potentially help reduce the risk factors. Feeding contaminated offal to domestic pets could facilitate the transmission of the infection to humans. Improper kitchen and personal hygiene practices too could lead to the infection in humans (Bolton et al., 1985).

2.3 Metagenomics

In the past decade, next generation sequencing (NGS) has been used in many fields to investigate antimicrobial resistance, outbreak investigation, food authenticity, etc. The technology is rapidly advancing in quality with a cost reduction while influencing food microbiology. In food microbiology, NGS used to determine the whole genome sequence of any single cultured isolate (whole genome sequencing) and to generate sequences of microorganisms present in biological samples (Jagadeesan et al., 2019).

Applying metagenomics to enhance quality and safety of food is still at its early stages. Metagenomics serve as a platform to predict the emergence of pathogenic microorganisms and identify unknown microbiota (Jagadeesan et al., 2019).

The traditional methods for pathogen detection culture methods and microscopy, though useful, are subjected to limitations such as specific culture requirements of most species and genera. Moreover, these methods fail to assess the microbiome at the ecological level. The modern techniques used such as nucleic acid amplification or immunoassays are also subjected limitations such as the ability to only detect a single or a few pathogens at a time. Bacterial populations are subjected to stress when the surrounding environment changes. The stress leads to reorganization of microbes, which impacts the persistence of foodborne pathogens in food systems. Hence, metagenomics is used to investigate the influence whole microbial communities on the existence of pathogens (Yang et al., 2016).

Metagenomics is composed of two sequencing strategies: amplicon sequencing using 16s rRNA as a phylogenetic marker and shotgun sequencing (captures the complete breadth of DNA present in a sample). The use of 16s rRNA gene as a phylogenetic marker has been found to be efficient and cost effective to analyse microbes (Rausch et al., 2019).

Improvements in DNA sequencing techniques and barcoded pyrosequencing application has uplifted 16S rRNA profiling application. NGS technologies (454 and Illumina sequencers) 16S rRNA amplification primers targeting hypervariable regions. Nine hypervariable regions are

present in 16S rRNA genes (V1–V9) that indicate varying sequence diversity in different bacteria. hypervariable regions V2 (nucleotides 137–242), V3 (nucleotides 433-497) and V6 (nucleotides 986–1043) which are of highest heterogeneity providing higher ability to distinguish between groups of bacteria (Shah et al., 2011).

A study was conducted to investigate the effect of processing water and processing time on the microbial diversity of yellow-feathered broiler carcasses at selected stages of slaughter (Wang et al., 2019). In that study, the V3-V4 region of the 16S RNA gene was targeted. Proteobacteria and Fermicutes were found to be the most dominant bacterial phyla on the carcasses and water in the chiller tank. The water in the scalding tank showed a high abundance of Firmicutes and Deinococcus-Thermus. The boiler carcasses showed high abundance of Escherichia-Shigella and Streptococcus at the genus level. However, they showed a reduction after being washed and chilled. The alpha diversity metrics (Chao 1, Shannoneven and Shannon) revealed that bacterial community structures became more complex at late stages of processing. The PCoA analysis depicted that the bacterial clustering was significantly separated between water from the scalding tank and carcasses indicating a limited effect of scalding water on the bacteria present on broiler carcasses (Wang et al., 2019).

A study was performed to detect the pathogenic bacteria present in a beef production process. The presence of pathogenic bacteria was investigated during the stages of cattle entry to feedlot and exit from feedlot as well as in cattle transport trucks, abattoir holding pens, and the end of the fabrication system (Yang et al., 2016). The major phyla observed at arrival, exit, holding pen, and market-ready was Proteobacteria, followed by Actinobacteria, Firmicutes, and Bacteroidetes with varying proportions. An extremely high proportion of Bacteroidetes was observed in truck samples. Cyanobacteria and Chrysiogenetes were the other major phyla present in truck samples. Shannon diversity index of arrival, exit, and holding pen samples showed no statistical difference, but was higher than that of truck and market-ready samples. The similarity between pathogen abundance in arrival and exit samples may have been the result of homeostasis of the microorganisms in the feedlot environment. The similarity in the pathogen proportions between arrival and holding pen samples may be due to the existence of similar microbe diversities in both the samples. The reduced diversity in microbes observed in market-ready samples indicate the impact of antibacterial techniques used in the beef abattoir (Yang et al., 2016).

2.4 Mycotoxins

The presence of harmful organic compounds in foodstuffs is a major food safety concern worldwide (Cavus et al., 2018).The occurrence of mycotoxins in food and feed threatens the health of animals and humans due to its toxic, carcinogenic and mutagenic effects (Iqbal et al., 2014). Mycotoxins are naturally occurring secondary metabolites of fungi and are found to contaminate around one fourth of the world's crops (Al-Taher et al., 2013)

As mycotoxins could readily affect humans via the animal food chain, they have attracted great attention worldwide due to their health concerns (Wang et al., 2018). The most important mycotoxins on the basis of their occurrence and toxicity are AFs, trichothecenes, ochratoxins and ZEAs (Iqbal et al., 2014). Aflatoxins produced by *Aspergillus parasiticus* and *Aspergillus flavus* are considered to be highly toxic. They are classified as a group I carcinogen (Wang et al., 2018). The most acute and toxic class of aflatoxins is AFB_1. (Iqbal et al., 2014). Trichothecenes are a group of structurally related fungal secondary metabolites. The most significant mycotoxins of this group are produced by the species Fusarium, such as T-2 toxin, which is the most toxic in the group, although DON is the most frequently found. Metabolites of DON are common food contaminants in meat, eggs and milk from livestock animals (Zou et al., 2012). Its most significant effect is the estrogenic effect which was observed in children with precocious sexual development (Wang et al., 2018). Thus, trichothecene contamination of feed and feed ingredients is a major problem the world is faced with (Zou et al., 2012). Ochratoxins are secondary metabolites produced by different species of Aspergillus. The most commonly found in food is OTA that is known to contaminate animal feed leading to the presence of OTA in the meat and meat products of those animals. Meat has been suspected as the main source of OTA in human diet (Denle & Perez, 2010). OTA producing moulds are capable of growing at low water activity and temperature, which make common preservation technologies not effective in preventing mould growth.

Table 2.3 Natural occurrence of different mycotoxins in offal meat

Offal product	Mycotoxin contamination level					Reference
	AFB1	DON	ZEA	T-2	OTA	
Chicken liver	ND	ND	40.00-74.00 µg/kg	-	-	(Wang et al., 2018)
Chicken heart	ND	ND	49.30-87.50 µg/kg	-	-	
Chicken gizzard	ND	ND	39.90-84.90 µg/kg	-	-	
Chicken liver	0.57-3.80 µg/kg	-	-	-	-	(Sineque et al., 2017)
Chicken gizzard	0.68-2.12 µg/kg	-	-	-	-	
Beef liver	0.00-20.00 µg/kg	-	-	-	-	(Hassan et a., 2014)
Beef kidney	3.80-24.00 µg/kg	-	-	-	-	
Beef kidney	0.04	-	-	-	-	Oyero & Oyefulo (2010)
Beef heart	0.03	-	-	-	-	
Chicken liver	0.30-16.36 µg/kg	-	-	-	-	(Amirkhizi et al., 2015)
Chicken liver	-	-	-	-	4.06-7.68 µg/kg	(Al, 2018)
Chicken gizzard	-	-	-	-	1.89-2.26 µg/kg	
Buffalo liver	-	-	0.10-4.34 ng/g	-	-	(Mahmoudi, 2014)
Pork kidney					0.54 ng/g	(Curtui et al., 2001)
Pork liver					0.16 ng/g	

ND – Not detected

The daily maximum tolerable OTA dose has been reported to be 16.00 µg/kg (Kaynarca et al., 2019). ZEA is produced by *Fusarium* species (*F. graminearum, F. culmorum, F. poae and F. sporotrichioides*), toxin producing fungi mostly prevalent in the northern temperate regions. ZEA is an estrogenic mycotoxin, which do not result in fatal toxicities but will lead to reproductive abnormalities, mainly in swine. ZEA is produced by some fungi that also produce tricothecenes. Therefore, they may be present with DON. In mice and swine fed with pure ZEA, symptoms of estrogenic syndrome have been reported. Humans may be directly exposed to the ZEA toxin via drinking water, inhalation or through the consumption of meat of infected animals (Pleadin et al., 2015).

In a study conducted to investigate the risk of exposure to AFs, DONs and ZEAs in chicken meat in China, it was found that all the heart, gizzard and liver samples were free from AFs and DONs (Wang et al., 2018) (Table 2.3) In contrast, Amirkhizi et al., (2015) reported the presence AFB_1s in chikcen livers (0.30-16.36 µg/kg) (Table 1). Iqbal et al. (2014) found that in layers chicken breed the highest levels of total aflatoxin (3.23 ± 0.82 µg/kg) and AFB_1 (2.98 ± 0.76 µg/kg) were both present in the chicken liver samples compared to those of chicken wings,

chest and legs. They further reported that in broiler chicken also the same trend was observed for total AF (3.40 ± 1.01 µg/kg) and AFB_1 (2.64 ± 0.58 µg/kg) in the liver samples compared to other samples (Table 2.3). However, in domestic chicken AFB_1 was not observed in any of the samples (liver, wings, chest and legs). The AF incidence in the layers and broiler breeds of chicken maybe a result of different feeding practices. Literature supports the contamination of poultry feed by mycotoxins (Zinedine et al., 2007; Rosa et al., 2006).

Sineque et al. (2017) has also reported the presence of AFB_1s in chicken livers (0.57-3.80 µg/kg) as well as in chicken gizzard (0.68-2.12 µg/kg). A higher incidence of AFB_1 was observed in the liver (Table 2.3).This is due to the intense metabolism AFB_1 undergoes in the liver (Bailly & Guerre, 2009).

Hassan et al. (2013) has reported the presence of AFB1 in sheep carcasses obtained from 3 abattoirs in Egypt (41.69 ± 2.53, 32.80 ± 2.14 and 26.85 ± 1.79 µg/kg). Poor hygienic measures in handling of meat is depicted through the high incidence of mould on the carcass surfaces (Hassan et al., 2013). Though there are no recent studies published on mycological evaluation based upon sheep offal, these findings indicate the possibility for cross contamination of sheep offal during processing. Another study from Egypt focussed on beef meat and offal revealed AFB_1 ranges of 0.00-20.00 and 3.80-24.00 µg/kg in beef liver and kidney, respectively. Among all studied products, beef kidneys were found to have the highest level of AFB_1 Hassan et al., 2014) (Table 2.3). AFB_1 is excreted via the urinary pathway and this explains the high incidence in the kidney (Li et a., 2018). However, Oyero (2010) had reported the presence of AFB_1 in kidney (0.04 µg/kg) to be less than in the liver (0.07 µg/kg) but higher than in the heart (0.03 µg/kg). The AFB_1 contents and the total AF contents obtained for the beef offal were much less than the permissible in African standards 5 mg/kg for AFB_1 and 20 mg/kg for total AFs in food. In ruminants, upon the ingestion of AF contaminated feed, part of it will be degraded into aflatoxicol by ruminal flora. The rest will be absorbed via the digestive tract through passive diffusion and hydroxylate in the liver to form AFM_1. AFM_1 will either enter the blood circulation or get excreted through bile. AFM_1 in the blood circulation maybe present in urine or milk (Fink-Gremmels, 2008).

Wang et al. (2018) reports that DON was absent in the chicken liver, heart and gizzard samples that were examined. Pigs fed with feed artificially contaminated with 10.31 mg/kg DON were found to have 3.38 ± 0.46 and 3.49 ± 0.32 µg/kg of DON in liver and kidney samples, respectively. DON was absent in all heart samples (Wu et al., 2013).

Though there was no significance difference between the two sets of results in an earlier study with kidneys of DON-exposed pigs reported to have a higher DON content than the livers (Dänicke et a., 2004). Liver is responsible for the DON metabolism as well as detoxification. Previous reports suggest that DON conjugates with glucorinide and gets detoxified via mammalian UDP-glucuronosyl-transferase in the liver (Wu et al., 2013).Hence, the liver is an organ susceptible to DON. Hassan et al. (2013) found sheep carcasses to have 3.33 – 10.00% *Fusarium*. Since Fusarium produce DON this finding suggests the possibility of DON contamination of sheep meat (Pleadin et al., 2015). Ruminants however, are not highly susceptible to DON as rumen flora converts it to less toxic DOM (the de-epoxidized metabolite of DON). Studies have shown that cattle is capable of tolerating diets with 8.5 mg/g DON for many weeks without being subjected to major health effects (Fink-Gremmels, 2008).

Wang et al. (2018) reported that the liver, heart and gizzard had ZEA ranges of 40.00–74.00, 49.30-87.50 and 39.90-84.90 µg/kg, respectively. Overall, the liver, heart and gizzard samples had similar ZEA contents (Wang et al., 2018) (Table 2.3). A study from Iran using buffalo meat revealed that most of the liver samples (68.57%) were contaminated with ZEA compared to milk (21.42%) and meat (41.42%). ZEA range in the buffalo were found to be (0.10-4.34 ng/g) and (0.10-2.50 ng/g) in the samples analysed (Mahmoudi, 2014) (Table 2.3). The higher incidence of ZEA in liver samples may be due to the hepatic biotransformation pathways occurring in animals. They are the binding of ZEA and reduced metabolites with glucuronic acid and hydroxylation which results in α-zearalenol and β-zearalenol (Bailly & Guerre, 2009). ZEA is known to be converted to the hydroxy-metabolite α-zearalenol by the ruminal flora. Alpha zearalenol is known to have a higher oestrogenic potential compared to Zearalenone, yet due to its inter conversion into less toxic β-zearalenol in the cattle are known to be less susceptible to adverse effects of ZEA (Fink-Gremmels, 2008).
The same study showed that the ZEA content observed during autumn was much higher than during summer in the raw animal origin food (buffalo milk, meat and liver). This could be explained by the high amounts of ZEA produced by moulds at low temperatures and high humid environments (Krnjaja et al., 2009).

A study from Jordan revealed that 100% of the chicken and gizzard samples were positive for OTA. The OTA levels of chicken liver samples (4.06-7.68 µg/kg) were found to be higher than in the chicken gizzard samples (1.89-2.26 µg/kg) considered in the study. This could be explained by the OTA detoxification which occurs at the hepatic level into minor metabolites such as 4-hydroxy-ochratoxin (Bailly & Guerre, 2009). (J∅ rgensen, 1998) has reported that 94.6% of pork kidneys were positive for OTA levels > 0.02 ng/g. He further concluded the

maximum OTA content to be 15 ng/g. This maybe a result of the re-absorption of OTA in kidney tubules facilitated by anionic transporters. In contrast, Curtui et al. (2001) reported to find OTA amounts of 0.54 and 0.16 ng/g in pork kidneys and livers respectively (Table 2.3). The reason for lower levels OTA present in porcine kidneys may have been due to the initial OTA contamination in feed.

Compared to monogastric animals, ruminants are known to develop mycotoxicosis less frequently due to its rumen flora acting as a first line of defence towards mycotoxins (Dänicke et a., 2004; Fink-Gremmels, 2008). A rapid conversion of OTA in to the less toxic ochratoxin α occurs within ruminants by forestomach flora. In healthy cattle up to 12 mg/kg of OTA feed could be inactivated. However, extreme changes in the feed composition and the presence of high amounts of protein-rich concentrates in the feed could affect the cleavage capacity of the gut microbes (Fink-Gremmels, 2008). This may lead to the presence of OTA in ruminant milk and tissues.

Overall, results indicate that offal are prone to mycotoxin contamination and liver has a higher affinity towards accumulation of mycotoxins. The ranges of the different mycotoxins present in different organs may vary from one geographic location to another due to environmental factors such as the degree of contamination of the feed, humidity and temperature.

The above mentioned results confirm the occurrence of mycotoxins in meat from animals and the need to investigate these further. Though many studies have been published related to the mycotoxin presence in meat not many studies have been focussed on offal. Furthermore, many of such published research is focussed on poultry offal (Wang et al., 2018); Amirkhizi et al., 2015; Al, 2018). This clearly indicates the need to investigate red meat offal for the presence of mycotoxins.

2.5 Decontamination of meat

2.5.1 Physical decontamination of meat

Microorganisms threaten the safety and quality of food worldwide. The susceptibility of consumers to foodborne diseases upon the consumption of contaminated food has raised awareness for microbiological safety of foodstuff. Microbiological safety has shaped the manner in which food is processed, manufactured, packaged, distributed, and prepared. Red meat, as wells as other food, has been documented as a vehicle for the transmission of foodborne pathogens. This has given a rise to the need to develop and implement techniques to eliminate risks associated with food production and ensure the quality and safety of food. A technology

that can result in the elimination of a microorganism without leaving products of microbial metabolism or need to use antimicrobial additives is called "strategy of physical decontamination" (Bacon, 2005). Some of the commonly used techniques to prevent contamination are trimming, water spray washing and pasteurisation.

2.5.1.1 Trimming

Rapid spoilage of meat products may take place if faeces or ingesta contaminate red meat tissues during slaughtering and processing. Trimming is a process where contaminated tissues are mechanically removed with the aid of a knife. Reagan et al. (1996) reported that carcass trimming significantly reduced faecal contamination as well as APC and biotype 1 *E. coli* counts by 1.30 and 1.60 log CFU/cm^2. Phebus et al. (1997) reported that trimming of artificially contaminated beef tissue surfaces reduced counts of *E. coli* O157:H7, *Salmonella typhimurium (S. typhimurium)*, and *L. monocytogenes* by 3.10, 2.70 and 2.50 log CFU/cm^2, respectively. A study performed using beef round, brisket and cold surfaces that were either inoculated with a faecal inoculum or left un-inoculated revealed that counts of *S. typhimurium*, *E. coli* O157:H7, APC, *Enterobacteriaceae*, total coliform, thermotolerant coliform, and biotype I *E. coli* counts reduced significantly (2.60–4.30 log CFU/cm^2) (Castillo et al., 1998). It has been suggested that the physical removal of contaminated tissues may prevent the translocation of microorganisms to contaminant free surfaces. Though these results may depict trimming as an excellent mean of reducing bacterial populations on meat, this method fails to achieve the same level of reductions during a normal slaughter process. Furthermore, as this method focusses only on visible contamination and non-visible bacterial contamination cannot be removed by trimming (Castillo et al., 1998).

Gill & Badoni (1997) conducted a study with broiler carcasses to investigate the effect of trimming and washing (water with 0.50 to 1.00 mg/kg of residual chlorine at 20–25°C temperature) on reduction of bacteria. The study revealed that trimming lead to a significant decrease in aerobic mesophiles, but a higher count of *E. coli*. According to the study's findings washing was found to be more effective than trimming. Trimming is also a process which requires successful identification of contaminated surfaces, appropriate operational sanitation to prevent cross contamination and high levels of training and experience of involved employees (Bacon, 2005).

2.5.1.2 Water spray washing

Water spraying systems are mostly automated and therefore less prone to human error. Water spray washing may be superior to trimming due to its ability to impact bacterial population densities regardless of the presence of visually identifiable contamination. The reduction of bacterial populations will be determined by the tissue conditions. In fresh carcases, microbiological contamination contact time is short, but after being chilled microbes may be present on meat surfaces for long periods of time. Attachment of bacteria to meat surfaces depends on time and has two stages. Firstly, the attachment is reversible due to van der Waals' forces causing the binding of the microorganism to the carcase surface. Longer contact period lead to the second stage where the contact between the bacteria and the surface is associated with strong, irreversible binding involving polymer production and glycocalyx development and the subsequent formation of a biofilm. The second phase of attachment helps bacteria survive on meat surfaces by protecting them from environmental stresses. Pressure and temperature are two major parameters that control the efficacy of water spray washing (Bacon, 2005).

Kotula et al. (1974) reported a study that when beef forequarters were treated with hyperchlorinated (200 ppm) water applied at pressures of 411.90 and 2412.40 kPa for 30 seconds, the APC values were reduced by 1.20 and 2.60 log CFU/ cm^2, respectively, after 45 minutes of spray-washing . In a study conducted by Anderson et al., (1975) to study the impact of different pressures (343.20, 686.50, 1382.70, and 2755.70 kPa) on beef surface to remove *Rhodatorula rubra* , the authors found that as the pressure increased from 343.20 to 2755.70 kPa, the removal of *R. rubra* from the surfaces increased from 84.00 to 94.00%. Reductions of APC and streptomycin-resistant *E. coli* ranged from 1.70–2.20 and 1.80–2.30 log CFU/cm^2, respectively, in beef brisket adipose tissues inoculated with faecal slurry when the spray washing pressure was increased from 276 to 2758 kPa (Gorman et al., 1995). In spite of these results, use of high water pressure may lead the bacteria present on the surface to translocate to internal tissues and increase the water uptake in treated tissues (Anderson et al., 1975). When Dixon et al. (2019) subjected beef carcasses to high pressure water treatment (15 L of hot water at 90°C and 53 L of cold water at 4°C for each carcass during 10 seconds treatment) there were no reductions in the counts of aerobic mesophiles, *Enterobacteriaceae* and *Pseudomonadaceae*. Instead, a significant increase in numbers were observed in the bacteria, which was contradictory to results from previous studies (Crouse et al.,, 1988; Gorman et al., 1995; Kotula et al., 1974). This may indicate potential migration of the bacteria to the inner tissues due to pressure effect.

With regard to ensuring microbiological safety of red meat, many reports have been published related to spray washing linked to water temperature (Dorsa et al., 1996; Gill & Jones, 1998; Smith 1992; Delmore et al., 1998). This highlights the synergistic effect that could be achieved by this technique combination (Bacon, 2005).

In a study where ovine surface tissues were investigated for the effect of temperature on the reduction of aerobic bacteria results revealed that 82.20^{0}C spray washing reduced the total count by about 3.00 log CFU/cm^2 (Dorsa et al., 1996). Gill & Jones (1998) reported that when pork carcasses were spray washed at 85^{0}C, coliform as well as total aerobic counts were reduced by > 2.0 log CFU/cm^2. In a study where beef briskets were exposed to water at 80^{0}C for 10 and 20 seconds best reductions for *E. coli* (from 6.78 to 3.26 log CFU/cm^2), *Salmonella* (from 6.88 to 3.28 log CFU/cm^2) and *Listeria* spp. (from 6.20 to 3.32 log CFU/cm^2) were observed when treated for 20 seconds (Smith, 1992). However, as these are harmful foodborne pathogens, it is essential to ensure they are completely eradicated from meat. A main concern related to usage of hot water as a decontamination technique is the bleached appearance it may impart to meat (Gill & Badoni, 1997).

Delmore et al., (1998) reported that when treated with 80^{0}C water, beef adipose tissues that were inoculated with high levels of *E. coli* (ATCC 11370) showed reductions from 7.90 and 6.80 log CFU/cm^2 to 5.70 and 4.90 log CFU/cm^2 in aerobic plate counts and *E. coli* counts, respectively. At low inoculation levels, the APCs and coliforms were reduced from 3.70 and 3.40 log CFU/cm^2 to 3.50 and 2.50 log CFU/cm^2, respectively. The reductions achieved at low inoculation levels were not significant compared to the high inoculation levels. This questions the ability of these treatments to completely eradicate pathogenic microbes.

2.5.1.3 Pasteurization

Steam pasteurization includes water removal from the surface of carcase to minimize the steam barrier, 'saturated steam' exposure of carcass surfaces and finally cooling the carcase surface to reduce the heat effect on the product's colour (Bacon, 2005).

Gill and Bryant (1977) reported that the mean difference obtained from 50 carcase surfaces in aerobic plate counts before and after treatment of steam pasteurization (commercial) was about 1.00 log CFU/cm^2. Nutsch et al. (1997) showed that when pre-rigor beef contaminated with faecal slurry were subjected to steam pasteurization for 15 seconds counts of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* were reduced by 3.50, 3.70, and 3.40 log CFU/cm^2, respectively. In a study where sheep carcasses were treated with steam pasteurization (water

temperature ≥ 82 °C) a clear reduction of *Enterobacteriaceae* and *E. coli* was observed after treatment. The number of samples positive for *Enterobacteriacae* and *E. coli* dropped from 38% to 12% and 30% to 12%, respectively after treatment with steam pasteurization (Hassan et al., 2015). In sheep carcasses hot water pasteurization (mean temperature 72-86^{o}C) was found to reduce the counts of *E. coli*, Enterobacteriaceae, and APC by 0.70, 1.00, and 0.90 log CFU/1000 cm^2 , respectively, compared to the untreated carcasses (Omer et al., 2015).

Although the above findings suggests pasteurization as a promising technique to reduce pathogenic bacteria from carcass surfaces, environmental factors such as growth temperature, exposure to previous stress may develop an ability in microbes to resist thermal stress. In *E. coli* O157:H7, *S. typhimurium* and *L. monocytogenes* thermal resistance has been documented (Bacon, 2005).

2.5.2 Chemical decontamination of meat

Usually the carcass is contaminated by enteric pathogens during the process of slaughter and the locations may vary. Hence, chemical treatment would be required for all of the meat surfaces. Different type of chemicals have varying levels of effectiveness towards bacterial inhibition. However, if the bacteria are not properly exposed to the right chemical, a complete inhibition may not be effective. If bacteria have been attached to the meat surface for a long period of time this may reduce their sensitivity towards chemicals (organic acids) due to the meat surface's buffering capacity. If chemical decontaminants are to be utilized in a HACCP system, validation of control is needed. This would be challenging due to the difficulty to create conditions that would occur on a carcase during a commercial slaughter operation (Bacon, 2005).

2.5.2.1 Chlorine

Kotula et al., (1974) reported that beef forequarters treated with chlorinated water at 200 ppm exhibited total aerobic counts log reductions of 1.50 and 2.30 when sampled at 45 minutes and 2.40 and 3.10 when samples at 24 hours post wash. This was in contradiction to the findings of Anderson et al., (1977) who reported no significant reductions in total viable counts were observed when meat was treated with 200 ppm sodium hypochlorite.

Chemical treatments, including different chlorine forms, have been reported to have positive effects on meat decontamination. Castillo et al., (1999) revealed that when pre-rigor beef carcasses were exposed to acidified sodium chlorite (ASC) with phosphoric acid *E. coli*

O157:H7 and *S. typhimurium* were reduced by 3.8–3.9 log, and 4.5–4.6 log with ASC prepared with citric acid. In contrast, Gill & Badoni (2004) found ASC to have minimal effect on reducing *E.coli*, coliforms and aerobic bacteria on chilled beef surfaces. Cutter & Rivera-Betancourt (2000) reported that cetylpyridinium chloride (CPC) (1% for 15 seconds treatment time) is effective in reducing *E. coli* O157:H7 and *S. typhimurium* present on lean beef from 5.00–6.00 log CFU/ cm^2 to undetectable levels.

For beef lean tissue inoculated with a mixture of *E. coli* 0157:H7, *Salmonella typhimurium, C. jejuni,* and *C. coli*, sodium hypochlorite at 600 ppm resulted in higher log reductions (1.80 to 2.20 log CFU/ cm^2) than at 100 ppm (1.40 to 2.00 log CFU/ cm^2). However, at 100 ppm sodium hypochlorite was more effective in reducing APC, *E. coli*, and coliforms in the range of 2.20 to 2.40 log CFU/ cm^2 than at 600 ppm. These results indicate that the sensitivity of different bacteria towards chemical disinfectants may vary independent of its concentration.

When a chicken drumstick with skin and a skinless chicken breast meat were inoculated with *L. monocytogenes* and treated with 1200 ppm of ASC (0.5 minutes attachment time) bacterial counts were reduced to 1.40 and 0.70 log CFU/ml, respectively. As the attachment period was extended up to 210 minutes, no bacterial resistance to the treatment was exhibited in the drumstick. However, in the chicken breast as the attachment period was extended from 20 minutes to 210 minutes bacterial resistance was exhibited. In the same study when the chicken drumstick and the breast meat were inoculated with *S. typhimurium* and treated with 1200 ppm of ASC after 0.5 minute of attachment, the bacterial counts observed were 1.80 and 0.90 log CFU/ml, respectively. In the drumstick, no resistance was exhibited when the attachment periods were extended to 20 minutes and 210 minutes. In the breast meat resistance was exhibited when attachment time was extended from 0.5 minutes to 20 minutes. When the attachment time was further extended to 210 minutes there was no change in resistance was observed (Ilhak et al., 2018).

2.5.2.2 Organic acids

Organic acids have been used to decontaminate carcass surfaces in many research studies (Ockerman et al., 1974; Anderson et all., 1977; Yoder et al., 2012; Cil et al., 2019, Ilhak et al., 2018). It is generally accepted that the undissociated organic acids molecules have antimicrobial activity. Many weak acids in the undissociated form have the ability to accumulate in the cytoplasm by penetrating the cell membrane. If the pKa of the acid is less

than the intracellular pH the acid will release a proton via dissociation and result in cytoplasm acidification (Bacon, 2005).

Ockerman et al., (1974) suggested that microbes count is reduced when lamb carcasses are treated with varying concentrations of lactic and acetic acids. Treating meat with acetic acid (3%) was reported to reduce bacterial numbers on meat by 2.60 log cycles (Anderson et al., 1977). In certain occasions a certain level of discolouration of tissue or fat surfaces occur when organic acids come into contact with hot carcass surfaces. Less discolouration occurs at low concentrations. However, the damage caused to the hot carcass surface product quality loss limits the usage of high organic acid concentrations in industrial applications (Bacon, 2005).

Yoder et al. (2012) inoculated meat samples with a faecal slurry (mixture of *E. coli* 0157:H7, *S. typhimurium*, *C. jejuni*, and *C. coli)* was treated with rinses of citric acid, lactic acid and acetic acid at concentrations of 1, 2 and 5% (applied at 276 kPa, 30.50 cm away from the meat surface with a 15 seconds application time and five minute dwell period) greater reduction in bacterial numbers was observed with increasing concentration. At 5% the range of reductions exhibited by lactic acid, acetic acid and citric acid were 3.30 to 5.60 log CFU/ cm^2, 2.40 to 4.90 log CFU/cm^2 and 3.70 to 4.70 log CFU/ cm^2, respectively. The acids at 2% concentration seemed more effective in reducing the *Campylobacter* spp. numbers and lactic acid showed a higher reduction (6.36 log CFU/ cm^2) than citric acid (4.79 log CFU/ cm^2) and acetic acid (5.11 log CFU/ cm^2). On the contrary, *Salmonella* spp. was effectively reduced at 5% acid concentration. Lactic acid had the highest reduction effect of 4.96 log CFU/ cm^2. In another investigation, the effect of lactic acid on *C. jejuni* present on chicken skin at 2% and 3% concentrations were more effective at 22^0C compared to 50^0C on day zero of storage. However, 4% lactic acid at both treatment solution temperatures reduced the bacterial counts to undetectable levels. All lactic acid concentrations (2, 3 and 4%) treated at both temperatures showed a reduction in Campylobacter counts to undetectable levels after one, three and five days of storage at 40^0C (Cil et al., 2019). In a separate study after a 0.5 minutes attachment period in chicken drumstick and breast meat, a higher reduction in *L. monocytogenes* was observed when treated with 4% compared to 2%. However, when the drumstick was treated with lactic acid (2% and 4%) and the breast meat treated with 4% lactic acid and the attachment time was extended from 20 minutes to 210 minutes, a bacterial resistance was observed. Chicken drumsticks inoculated with *S. typhimurium* also had a higher reduction in numbers when treated with 4% acetic acid. Resistance was shown at both lactic acid concentrations when the bacterial attachment period was lengthened from 20 to 210 minutes. *S. typhimurium* in chicken breast on the other hand showed higher reduction in numbers when treated with 2%

acetic acid. Resistance was also exhibited when attachment time was extended from 0.5 to 20 minutes (Ilhak et al., 2018).

2.5.2.3 Ozone

When beef brisket samples artificially contaminated with faecal paste containing streptomycin-resistant *E. coli* strain reductions of 2.70–2.90 log CFU/cm^2 and 2.50–2.60 log CFU/cm^2 were observed in total plate counts and *E. coli,* respectively (Gorman et al., 1995). However, Reagan et al. (1996) reported spraying 0.30–2.30 mg/l ozone on beef carcass surfaces did not result in significant reductions in aerobic plate counts (no more than 1.3 log10 CFU/cm^2). A similar finding was reported by Castillo, McKenzie, Lucia, & Acuff (2003) where hot carcass surface were treated with faecal smears containing rifampicin-resistant *E. coli* O157:H7 and *S. typhimurium* and treated with ozone solutions of 95 mg/L. Reduction numbers obtained showed no significant difference from reduction achieved by water washing. Similarly when beef tissue inoculated with pathogenic bacteria (*E. coli* 0157:H7, *S. typhimurium*, C. *jejuni*, and *C. coli*) was treated with ozone enriched water, it was not found to be more effective than tap water in reducing the pathogen numbers (Yoder et al., 2012).

2.5.3 Emerging decontamination techniques

2.5.3.1 Pulsed light

Pulsed light (PL) is a technique used to minimize the bacterial incidence and toxin production in food. The pulses generated in this method inactivate the microbes present at the food surface. The microbe's DNA absorbs ultraviolet (UV) light resulting in physico-chemical changes in its structure, disrupting the genetic information and causing cell death (Tomasevic et al., 2019). Efficacy of pulsed UV light treatment (1.27 J/cm^2) on vacuum packaged and unpackaged chicken breast meat inoculated with nalidixic acid- and streptomycin sulfate-resistant (NSR) *S. typhimurium* was investigated. For unpackaged meat the treatments which resulted in ~99% reduction (~2 log) were 15 seconds and 30 seconds, which were conducted at distances 5 and 8 cm (distance between sample and the UV strobe) respectively. For the packaged samples most efficient treatment reducing ~99% of the pathogenic *Salmonella* was the 30-s treatment at 5 cm. However, the study revealed that due to heat generation in the chamber a visual colour change would occur if 15 seconds was exceeded. Along with an increase in the fluence, the lethality of a treatment is found to rise. Maximum reductions of *E. coli*, *S. typhimurium* and *L. monocyotgenes* were observed in beef carpaccio at a fluence of 11.90 J/cm^2 compared to other treatments (0.70, 2.10, 4.20 and 8.40 J/cm^2). The study revealed a significant decrease in

yellowness and redness in beef due to PL. Furthermore, samples exposed to 8.40 and 11.90 J/cm^2 changed the odour significantly and was found less fresh (Hierro et al., 2012).When dried cured ham samples were inoculated with *L. monocytogenes* and exposed different fluences (2.10, 4.20 and 8.40 J/cm^2) maximum level of reduction was observed at 8.40 J/cm^2. Immediately after PL treatment at 8.40 J/cm^2 a significant in difference in flavour was observed by the panellists (Fernández et al., 2020).

Continuous exposure to UV light causes lipid oxidation in meat. Since PL is very intensive UV light lipid peroxidation could occur in meat which will result in meat deterioration (Tomasevic et al., 2019). In a study where salami (fermented sausages) inoculated with *E.coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes* and *S. aureus* were treated with PL the concentration of malondialdehyde (MDA) was used to predict occurrence of lipid oxidation. In samples from both vacuum and modified atmosphere packaged immediately after treatment (3 J/cm2 and 15 J/cm2), no significant MDA concentrations were determined. However, during cold storage MDA concentrations in both vacuum and modified atmosphere packaged samples were found to increase. A significant level of MDA compared to the first day of storage was only observed nine weeks after cold storage in both sample types (Rajkovic et al., 2017). In contrast to these findings, Keklik et al., (2010) reported a significant lipid peroxidation in chicken treated with PL (3.90 J/cm^2) immediately after treatment.

Though PL may seem as a promising novel technique for meat decontamination its surface treatment involving energetic photons could promote the formation of radicals, and oxidation which could lead to the formation of toxic chemicals imposing a negative impact on human health (Rajkovic et al., 2017). Rajkovic et al. (2017) revealed that the log reductions observed were affected by the time between inoculation and PL treatment. All PL treatments showed a reduction of about 2.20 log CFU/g of pathogenic organisms (*E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes* and *S. aureus*) when treated after one minute. A decreased efficiency in reducing the bacterial count was observed when PL was applied 30 minutes after inoculation which is likely due to the pathogen's stronger attachment to the meat surface. These findings suggest the need for stronger pulse treatments, which would affect the sensory quality of food and potentially promote the formation of toxic compounds. Based on the significant differences between log reductions, the best treatments for unpackaged samples were the 15 seconds treatment at 5 cm and the 30 seconds treatment at 8 cm, both of which resulted in ~2 log (~99%) reduction. However, considering the visual colour change caused by the heat generation in the chamber, the treatments should not exceed 15 seconds at either 5 or 8 cm. For the packaged

samples, the best treatment condition based on the significant differences in log reductions was the 30 seconds treatment at 5 cm, which resulted in ~2 log CFU/cm^2 (99%) reduction.

2.5.3.2 Irradiation

Irradiation is known as a decontamination technique which enhances the hygienic quality, extend shelf-life and minimize toxic residues by avoiding the use of nitrites and other preservatives of chemical nature. The use of irradiation in red meats have been allowed by the United States Department of Agriculture (USDA) and Food and Drug Administration (FDA) at maximum levels of 4.50 and 7.00 kGy for refrigerated and frozen meats, respectively in 1999 (Nam et al., 2017). Electron beam irradiation has been found to ensure the safety as well as the hygienic quality of duck meats. When frozen duck meat was subjected to electron beam irradiation, the D_{10} value (irradiation dose required to inactivate 90% of the microbial load) was found to be 0.47 and 0.51 kGy for *L. monocytogenes* and *S. typhi*, respectively. An et al., (2018) in a separate study investigated electron beam application use on commercial cold duck meat found the total aerobic bacteria and yeast and moulds were reduced by 3 and 6 log cycles, respectively. Moreover, D_{10} values of 0.65 and 0.42 kGy were found for *S. typhimurium* and *L. monocytogenes* respectively (An et al., 2017). The interactions between radiolysis products and components of the meat can potentially reduce the meat quality. Oxidative chemical changes in meat could be accelerated in the presence of hydroxyl radical (strong oxidising agent). Irradiation also speeds up lipid oxidation specially in aerobically packaged meats and give rise to characteristic off-odours (Nam et al., 2017). Vitamins such as thiamine, vitamin A and E are also affected by irradiation.

2.5.3.3 High pressure processing (HPP)

HPP is another emerging technology which enhances meat safety. This is usually applied as a post-packaging decontamination technique. HPP when applied at 400 to 600 MPa has been found to effectively control *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp. *S. aureus* (Nam et al., 2017). However, Clariana et al. (2011) had reported that when dry cured ham was treated with HPP at 600 MPa at 15 °C for 6 min the colour and sensory attributes were altered. An increase in brightness, hardness, odour intensity and chewiness were some of the observations.

2.5.4 Chitosan as a decontamination agent for food

Chitosan is the deacetylated form of chitin, which is amongst the most abundant natural polymers in living organisms (fungi, insects, and crustacea). Chitosan is known to have antibacterial properties which depend upon factors such as the pH, temperature, molecular weight, degree of deacetylation, etc. (Kanatt et al., 2013). Chitosan has been approved by the USFDA (2001) as generally recognized as safe (GRAS) (Kanatt et al., 2013). Chitosan has been tested on various meat products to determine its potential as a bio-preservative (Ouattara et al., 2000; Sagoo et al., 2002; Soultos et al., 2008). Numerous studies have also been conducted to study the antibacterial effect of different chitosan on pathogenic bacteria.

When chicken meat inoculated with *E.coli* (ATCC 25922) and *S. typhi* (KCCM 11862) was treated with chitosan at concentrations of 0.50, 1.00 and 2.00%, significant reduction in numbers of both bacteria was observed. The level of inhibition showed an increasing trend with increasing the concentration of chitosan. At 2% *S. typhi* and *E.coli* showed highest inhibition zones of 12.00 ± 1.50 mm and 12.30 ± 0.30 mm respectively (Kim & Kim, 2007).

Beef meat balls inoculated with *E .coli* O157:H7, *L. monocytogenes* and *S. typhimurium* (initial counts approximately 6.00 log CFU/g) were treated with 0.40% (w/w) shrimp chitosan solutions. By the 10th day bacterial counts of 4.40 ± 0.40 and 5.70 ± 0.40 CFU/g were observed for *E .coli* and *L. monocytogenes* respectively. However, chitosan was not found to be effective against *Salmonella*.

When four types of crab chitosan of different degrees of deacetylation were used to treat *E. coli* and *S. aureus*, the chitosan with the highest degree of deacetylation (86.02 ± 1.31%) was found to show the highest level of antibacterial activity (Huang et al., 2019).

E. coli (ATCC 25922) showed inhibition zones of 17.00 ± 0.50, 14.00 ± 0.30 and 10.00 ± 0.80 mm when treated with cuttlefish, crab and shrimp chitosan respectively. *S. aureus* (ATCC 25923) showed inhibition zones of 14 ± 0.5, 12 ± 0.0 and 9 ± 0.5 mm cuttlefish, crab and shrimp chitosan respectively (Hajji et al., 2015). Overall results suggested that cuttlefish chitosan with the lowest AD as well as lowest Mw showed the highest inhibitory level to both *S. aureus* and *E. coli*. Chitosan's ability to inhibit bacteria depends on the protonated NH_2 groups in chitosan which interact with the cell surface negative residues. Therefore, chitosan with a high positive charge (low AD) would exhibit high antibacterial activity (Hajji et al., 2015).

It has also been suggested that chitosan with high molecular weights could form a dense polymer film by wrapping the cell surface which would hinder the nutrient supply to the cell eventually causing cell death (Huang et al., 2019).

It has also been suggested that the antibacterial activity of chitosan maybe due to blocking of the nutrient supply to the cell which would hinder its metabolic activity (Tokura et al.,1996). Chitosan's metal binding capacity which inhibits the activity of various enzymes in the cell has also been suggested to lead to cell death (Darmadji & Izumimoto, 1996).

When treated with 10 mg/ml Blue crab chitosan *S. aureus* ATCC 43300 and *E. coli* ATCC 35218, *S. aureus* exhibited higher sensitivity (13.50 ± 0.71 mm) towards chitosan's antibacterial effect compared to *E .coli* (12.50 ± 0.71 mm) (Metin et al., 2019). A similar finding was observed in a separate study where minimal bactericidal concentration (MBC) values of 1300 ppm and 1400 ppm for *S. aureus* (ATCC 25922) and *E. coli* (ATCC 25923) respectively when treated with crab chitosan (Islam et al., 2011). The MBC value depicts the least concentration of an antibiotic required to cause 99% bacterial death (Islam et al., 2011).

Staphylococcus being a Gram positive bacteria is known to be more susceptible to chitosan's antibacterial activity as opposed to Gram negative bacteria such as *E. coli*. Gram positive bacteria contain techoic acids on its cell wall, which contribute to its negative charge. Similarly, in *E. coli* lipopolysaccharides (LPS) are present in the outer membrane (Raafat & Sahl, 2009).

In a previous study it was revealed that in Gram-positive bacteria the initial contact between the polycationic chitosan molecule and the bacterial cell is facilitated by the teichoic acids holding a negative charge (Raafat et al., 2008). This observation has been found to be consistent with the lower chitosan activity observed in Gram-negative bacteria where chitosan binds to LPS having a less impact on the cell envelope's dynamics (Raafat & Sahl, 2009).

Chen et al., (2002) reported that *S. aureus* (ATCC 27853) needed 36, 24 and 12 hours for concentration of shrimp chitosan at 1000, 2500, and 5000ppm, respectively to complete sterilization at deacetylation degree (DD) 90%. More positive charges are resulted with higher DD which in turn will interact with negatively charged bacteria. Therefore, higher DD will result in a higher growth inhibition. In the same study *E. coli* (ATCC 25922) (initial cell count 10^6 CFU/ml) also showed that inhibition was higher at 5000 ppm at a 90% DD. *S. aureus* showed higher inhibition at a higher molecular weight 6×10^5 compared to 6×10^4 kDa. In Gram positive bacteria such as *S. aureus* rather than a higher molecular weight a higher DD is found

to cause higher growth inhibition. On the contrary for gram-negative bacteria such as *E. coli* the molecular activity is found to have less or no inhibitory activity (Chen et al., 2002).

At present chitosan's ability as a preservation material for highly perishable food such as meat is under investigation. When treated with edible chitosan coating solutions, Harbin sausages, a local specialty in China, the growth of aerobic bacteria was controlled at 2% and 3% (w/v) edible chitosan solutions (stored at 23 ± 2 °C). After the 6th day of storage the total aerobic count (TAB) in 3% chitosan treated samples was found to be 4.55 log CFU/g which is less than the national standard of China for TAB (5.00 log CFU/g) (Dong et al., 2020).

In a study where the microbiological quality of beef coated with chitosan was investigated the total mesophilic aerobic counts of beef coated with chitosan and vacuum packed were much lower than that of uncoated vacuum packed beef. Moreover, *S. aureus* was found to completely disappear in the chitosan treated and vacuum packed samples whereas only inhibition was observed in the chitosan untreated vacuum packed beef (Duran & Kahve, 2020).These findings suggest that in addition to serving as an edible coat chitosan could also potentially contribute towards a longer shelf life due to its antibacterial properties.

Crab chitosan has been used in a study to investigate its effect on the shelf life of sushi, a popular ready-to-eat rice based food in Japan (Rachtanapun et al., 2018). Initial *S. aureus* load (approximately 3.00 log CFU/g) was found to reduce by 1.00 log after two days with treatment with 0.1% crab chitosan. This log reduction made the product acceptable for consumption whereas the chitosan untreated sushi (control) was found to have 3.90 log CFU/g of *S. aureus* making it unacceptable for consumption according to the Australian New Zealand Food Authority (ANZFA) guidelines.
Furthermore, no impact on sensory evaluation was observed due to the chitosan treatment (Rachtanapun et al., 2018). These findings suggest that chitosan could be utilized as a potential natural food preservative in the food industry. Therefore, the present study focuses on investigating the antibacterial effect of chitosan on meat inoculated with *S. aureus* and *E. coli* O157:H7, two major foodborne pathogens. Few studies have been conducted in recent years to study the antibacterial effect of chitosan in meat when combined with other antimicrobials or preservation techniques.

In a study where the shelf life of quail carcass was investigated when treated with lactic acid (LA), chitosan and modified atmosphere packaging (MAP) separately and in combination the total viable count (major determinant of poultry shelf life) was found to be controlled better by

MAP compared to LA. Chitosan was similar in effectivity to MAP. The study further revealed a synergistic effect between chitosan and MAP when treated in combination. However, the highest reduction in total viable count was observed when all three treatments (LA, chitosan and MAP) were used in combination (Ramezani et al., 2019). In another study when turkey breast samples were treated with 1% acetic acid and a mixture of 2% chitosan with 1% cumin after 9 days of storage at 40^0C the total viable counts were found to be lower than the proposed standard value (7.00 log CFU/g) as opposed to the control sample which exceeded this limit. The combination of chitosan and cumin was the most successful formulation to reduce the total viable count (Taheri et al., 2018).

When lean beef was inoculated with *E. coli* and *S. typhimurium* was treated with high molecular weight chitosan (HMWC), water soluble chitosan and chitosan oligosaccharides, HMWC was found to be the most effective against both the bacteria. Furthermore, HMWC was found to exhibit a synergistic effect with bacteriocin of *C. maltaromaticum* UAL307. This may have been due to the permeabilizing effect of the chitosan on the outer membranes of both bacteria. The synergistic effect was absent or weaker with the other two chitosan (Hu et al., 2019).

As consumers consider chemical additives to be a health risk, the food industry is inclined towards using natural antimicrobial agents to enhance food stability and protect from pathogens. As a result of microbial resistance towards currently available preservatives and consumer's preference towards chemical free food products a trend to study alternative inhibitors currently exist in the food industry (Cetin-Karaca & Newman, 2015). Chitosan may be a great alternative for this due to its antimicrobial as well as non-toxic properties. The current study investigates chitosan's ability to be used as a decontaminant on fresh meat. Though many findings related to the antibacterial effect of chitosan on *E. coli* O157:H7 and *S. aureus* have been reported chitosan's inhibitory action on these two pathogens when present on meat have not been investigated. Therefore, the present study focusses on chitosan's efficiency to decontaminate meat co-products inoculated with *S. aureus* and *E. coli* O157:H7.

Chapter 3: Materials and Methods

3.1 Microbiological analysis

3.1.1 Meat samples

Whole frozen samples of liver, kidney, heart, skirt, pizzle, testes, tail and tripe of sheep were obtained from Alliance Group, New Zealand.

Whole frozen samples of liver, kidney, heart, skirt, pizzle, testes and tail samples of sheep were obtained from sheep farms in Xinjiang and Ningxia provinces in China.

3.1.2 Aerobic plate count (APC)

Sub-samples were obtained from each of the meat samples. Meat samples were placed in stomacher bags (Nasco, Madison, USA) filled with 0.1% peptone (DIFCO Laboratories, Detroit, USA) + 0.85% NaCl (Merck, Darmstadt, Germany) (dilution fluid), usually 25 g in 225 ml and homogenized using a stomacher (IUL S.A., Barcelona, Spain). Ten-fold dilutions of the homogenate were prepared in dilution fluid. Volumes of 0.1 ml of the appropriate dilutions were dispensed to duplicate plates of plate count agar (Oxoid, Basingstoke, United Kingdom; Qingdao Hope Bio-Technology Co., Ltd, Qingdao, China). The inoculum was uniformly spread over the agar surface and allowed to dry for 15 minutes. Plates were inverted and incubated at 30^0C for 72 ± 3 hours in an incubator (Cohen et al., 2006). Colonies of the most suitable dilution were counted (30–300 CFU).

Staphylococcous aureus ATCC 25923 and a plate count plate inoculated with dilution fluid were used as the positive and negative controls respectively. For the preparation of the positive control, a loopful of *S. aureus* ATCC 25923 was diluted in 5 ml of sterile PBS. A meat sample (25 g) was inoculated with 1 ml of the *S. aureus* suspension. Then, the above mentioned APC procedure was carried out on the meat sample inoculated with *S. aureus*.

3.1.3 *E.coli* enumeration

Homogenates of excised meat samples were prepared in 0.1% peptone + 0.85% NaCl (dilution fluid) usually 25 g in 225 ml in a stomacher bag (Nasco, USA). Ten-fold dilutions of the homogenate were prepared in dilution fluid. Duplicate 3M^{TM} Petrfilm^{TM} *E.coli*/Coliform Count Plates (3M, Minnesota, USA) were placed on a flat surface and 1 ml of sample was dispensed onto the centre of the bottom film. The top film was slowly rolled down onto the sample and the sample was evenly spread. The Petrifilm plates were stacked clear side up and incubated at

37^0C for 18–24 hours in an incubator (Lee & Lee, 2016).Colony counting range was 15-150 as recommended by the manufacturer. *E. coli* NZRM 916 was used as the positive control. For the Chinese trials a positive control was not used due to the difficulty faced in resuscitating the *E. coli*. For the preparation of the positive control a loopful of *E. coli* NZRM 916 was diluted in 5 ml of sterile PBS. A meat sample (25 g) was inoculated with 1 ml of the *E. coli* suspension. Afterwards, the above mentioned *E. coli* test procedure was carried out on the meat sample inoculated with *E. coli*.

3.1.4 *Clostridium perfringens* Test

Homogenates of excised meat samples were prepared in stomacher bags (Nasco, USA) filled with 0.1% peptone + 0.85% NaCl (dilution fluid), usually 25 g in 225 ml using a stomacher (IUL S.A., Barcelona, Spain). Ten-fold dilutions of the homogenate were prepared in dilution fluid. Volumes of 0.1 ml of the appropriate dilutions were dispensed to duplicate plates of tryptose sulphite cycloserine agar (Oxoid, USA) supplemented with 98% D-cycloserine (Acros Organics, Belgium) according to the manufacturer's instructions. Plates were inverted and incubated anaerobically using anaerobic gas generating packs (MGC, Japan) at 37^0C for 20 hours in an incubator (Hauschild & Hilsheimer, 1974). The plates were observed for the presence of black presumptive positive colonies.

Clostridium perfringens NZRM 20 was used as the positive control. For the preparation of the positive control, a loopful of *Clostridium perfringens* NZRM 20 was diluted in 5 ml of sterile PBS. A meat sample (25 g) was inoculated with 1 ml of the *C. perfringens* suspension. Then, the above mentioned *C. perfringens* test procedure was carried out on the meat sample inoculated with *C. perfringens*.

3.1.5 *Listeria* Test

DuPont^{TM} Laterla Flow System *Listeria* Test was used. Sample (25 g) was placed in a stomacher bag (Nasco9, USA) and 225 ml of enrichment broth was added. Sample was homogenized for 30 seconds using a stomacher (IUL S.A., Barcelona, Spain) and the bags were incubated at 30 ± 0.5^0C for 40 hours in an incubator.

From the sample 400 µl was transferred to a plastic tube. The plastic tube was placed in a boiling water bath (100^0 C) for 10-15 minutes. Tubes were removed and allowed to cool to room temperature.

A test strip was inserted to the tube with the arrows facing downwards and allowed to develop for 10 minutes. The appearance of 2 red lines confirms the presence of *Listeria*.

Listeria monocytogenes ATCC 19111 were used as positive controls for the New Zealand and Chinese trials. For the preparation of the positive control, a loopful of *Listeria monocytogenes* ATCC 19111 was diluted in 5 ml of sterile PBS. A meat sample (25 g) was inoculated with 1 ml of the *L. monocytogenes* suspension. Afterwards, the above mentioned *L. monocytogenes* test procedure was carried out on the meat sample inoculated with *L. monocytogenes*.

3.1.6 *Salmonella* Test

For Salmonella detection samples (25g) were placed in stomacher bags (Nasco, USA) and sterile buffered peptone water (Oxoid, UK ; Qingdao Hope Bio-Technology Co., Ltd, China) (225 ml) was added, homogenized and the bags were incubated for 18-24 h at 37 ± 1^0C using an incubator (El-Aziz, 2013). After incubation, from the stomacher bags 0.1 ml was transferred to 10 ml of RVS broth (Oxoid, UK; Qingdao Hope Bio-Technology Co., Ltd, China). The broth samples were incubated at 42^0C for 24 ± 2 h in an incubator (Sen & Garode, 2016), and then 10 µl were streaked on to plates of Xylose Lysine Desoxycholate agar (XLD) (Oxoid, UK ; Qingdao Hope Bio-Technology Co., Ltd, China) and Brilliant Green Modified Agar (BGM) (Oxoid, UK ; Qingdao Hope Bio-Technology Co., Ltd, China) at 37± 1^0C for 18-24 h in an incubator. Presumptive Salmonella colonies (Pink colonies surrounded by bright red medium in BGM agar and red colonies with a black centre in XLD agar) were introduced to slants of Lysine Iron Agar (LIA) (Oxoid, UK ; Qingdao Hope Bio-Technology Co., Ltd, China) medium and Triple sugar iron agar (TSI) (Oxoid, UK ; Qingdao Hope Bio-Technology Co., Ltd, China) medium. Both media were incubated at 37± 1^0C for 18-24 h in an incubator (Hassanein et al., 2011). Serotyping was performed on presumptive positive colonies from TSI slant (purple throughout the tube with or without blackening). Two drops of 0.85% saline were placed side by side on a glass slide with a loop. A loopful of the pure culture was emulsified into both drops to give 2 dense, milky suspensions. One drop each of Salmonella polyvalent O antisera & polyvalent H antisera (Qingdao Hope Bio-Technology Co., Ltd, China) was added to each drop and the slide was tilted back and forth. Suspensions were examined for agglutination.

For the New Zealand trials *Salmonella Typhi* B8 and *E. coli* NZRM 916 were used as the positive and negative controls respectively.

For the trials in China Type B paratyphoid *Salmonella* CMCC 50094 and *E. coli* ATCC 25922 were used as the positive and negative controls respectively. For the preparation of the positive control, a loopful of Type B paratyphoid *Salmonella* CMCC 50094 was diluted in 5 ml of sterile

PBS. A meat sample (25 g) was inoculated with 1 ml of the *Salmonella* suspension. Afterwards, the above mentioned *Salmonella* test procedure was carried out on the meat sample inoculated with *Salmonella*. For the negative control a suspension of *E. coli* ATCC 25922 was similarly prepared and used to inoculate a meat sample (25 g).

3.1.7 *Campylobacter jejuni* Test

Sample (25 g) was added to 100 ml of Bolton broth medium (Oxoid, UK; Guangzhou hongquan biological technology co. LTD, Guangzhou, China) supplemented with aseptically defibrinated horse blood (Fort Richard, New Zealand; Guangzhou hongquan biological technology co. LTD, China) and Bolton broth supplement (Oxoid, UK; Guangzhou hongquan biological technology co. LTD, China) according to the manufacturer's instructions. The samples were enriched for 48 hours in a 42^0C in an incubator under microaerobic conditions (Paulsen et al., 2005). Afterwards enrichments were streaked onto modified Campylobacter blood free selective medium agar (CCDA) (Oxoid, UK ; Guangzhou hongquan biological technology co. LTD, China) agar plates and incubated at 37^0C for 48 hours under microaerobic conditions (Pezzotti et al., 2003) using microaerophilic gas generating packs (MGC, Japan) in an incubator. The plates were observed for the presence of white shiny colonies.

Campylobacter jejuni ATCC 2397 was used as the positive control for the New Zealand trials. A positive control was not used for the Chinese trials due inconvenience in sourcing the bacteria. For the preparation of the positive control, a loopful of *Campylobacter jejuni* ATCC 2397 was diluted in 5 ml of sterile PBS. A meat sample (25 g) was inoculated with 1 ml of the *C. jejuni* suspension. Afterwards, the above mentioned *C. jejuni* test procedure was carried out on the meat sample inoculated with *C. jejuni.*

3.2 Metagenomic analysis of sheep offal

3.2.1 Meat samples

Whole frozen samples of liver, kidney, heart, skirt, pizzle, testes, tail and tripe of sheep were obtained from Alliance Group, New Zealand.

Whole frozen samples of liver, kidney, heart, skirt, pizzle, testes and tail samples of sheep were obtained from sheep farms in Xinjiang and Ningxia provinces in China.

3.2.2 Sample preparation

The offal samples obtained from New Zealand and China were analysed at different times in both countries. Samples of each offal type weighing around 45-50 g were separately mixed with sterile phosphate-buffered saline solution (PBS) (1:10). Samples were mixed using a shaking incubator and 20 ml of PBS from the mixed sample was transferred to a centrifuge tube and centrifuged at 12000 rpm for 20 minutes at 4^0C. The supernatant was discarded and 1 ml of PBS was added to the remaining pellet and vortexed.

To investigate the richness as well as the species composition present in the offal samples high throughput sequencing of the 16S rRNA gene's V3-V4 region was used. Metagenomic DNA was extraction using the Macherey-Nagel NucleoSpin Soil kit (Macherey-Nagel, USA) according to the manufacturer's instructions. The extracted DNA was quantified using UV spectroscopy. The 16S rRNA gene's V3-V4 region was amplified using a pair of universal primers (338F5'-ACTCCTACGGGAGGCAGCAG-3', 806R5'-GGACTACHVGGGTWTCTAAT-3') The QuantiFluor ™ -ST Blue Fluorescence Quantification System (Promega) was used to detect and quantify PCR products. The PCR products were pooled and sequenced using Illumina Hiseq4000 platform by Shanghai Meiji Biomedical Technology Co., Ltd. (Shanghai, China). QIIME 1.9.1 was used for sequence classification annotation. Mothur software package (1.30.2) was used to obtain the readings for alpha diversity.

3.3 Mycotoxin analysis of meat samples

3.3.1 Meat Samples

Whole frozen samples of liver, kidney, heart, skirt, pizzle, testes, tail and tripe of sheep were obtained from Alliance Group, New Zealand.

Whole frozen samples of liver, kidney, heart, skirt, pizzle, testes and tail samples of sheep were obtained from sheep farms in Xinjiang and Ningxia provinces in China.

3.3.2 Mycotoxin extraction and analysis

A sub- sample (2g) of each meat type was added to a 50 ml centrifuge tube and 10 ml of 85% acetonitrile (Sigma-Aldrich, USA) solution was added and homogenized for 30 s at 2000 r/min using a high power dispersing instrument (IKA Works, Sdn Bhd, Malaysia). The samples were then centrifuged at 4000 rpm using a GPR Centrifuge (Beckman, USA) for 10 minutes at 4^0C and the supernatant was collected. The sample was again mixed with acetonitrile, homogenized

and centrifuged and the supernatant was collected (Chen et al., 2013).The supernatants were mixed and dried using a gentle stream of Nitrogen at 50^0C (DC12H, ANPFL Scientific, Shanghai, China). The concentrates were re dissolved in 1 ml of methanol: water (30:70, v/v) along with 0.1% formic acid and vortexed (XW-80A, Haimen, China) for 1 minute. Using a 0.22-μm filter (25 mm diameter, polyamide 6, organic phase; Tianjin, China) the solutions were filtered and analysed for the presence of mycotoxins (Deng et al., 2017). A Thermo Scientific Surveyor HPLC system was used for the toxin analysis of mycotoxin samples from New Zealand and China. A Hypersil GOLD column (5 μm, 100 mm × 2.1 mm) (Thermo Scientific, CA, USA) was used for the separation with a flow rate of 0.25 mL/min at 350C. The triple quadruple mass spectrometer used for the detection consisted of an electrospray ionization source. The mobile phase was water mixed with methanol and 0.1% formic acid and 5mM ammonium acetate and the volume injected was 5 μL. A sheath gas pressure of 35 au, auxiliary gas pressure of 15 au, spray voltage of 4500 V were used at a capillary temperature of 350^0C. The standards of AFB1, T-2 toxin, OTA, DON, ZEA and ZEN were from Enzo (USA). Stock solutions (10 μg/mL) of the standards were prepared using acetonitrile and stored at -20^0C. The stock solutions were diluted using a methanol / water (30/70, v/v) mixture containing 5 mM ammonium acetate to prepare the working standard solutions.

3.4 Investigation of the antibacterial effect of chitosan on meat

3.4.1 Meat samples

Whole frozen tripe samples purchased from Alliance Group, New Zealand were used.

3.4.2 Microorganisms

E. coli O157:H7 verotoxin negative strain number and *Staphylococcus aureus* ATCC 25923 were used. Frozen cultures were resuscitated using Tryptic Soya Agar (TSA) (Oxoid, UK) plates.

3.4.3 Inoculum preparation

A 10^8 CFU/ml suspension of each bacterium was prepared from an overnight culture in a TSA plate in phosphate-buffered saline (PBS) by adjusting the absorbance reading to 0.08-0.1 at 600 nm. The inoculum was prepared using a ten-fold dilution of the suspension using PBS.

3.4.4 Preparation of chitosan, acetic acid and ampicillin

Squid pen chitosan was obtained from Independent Fisheries Company (Christchurch, New Zealand) and crab chitosan (commercial) was obtained from Weseta International (Shanghai, China). Portions of each type of chitosan were gamma- irradiated at a dose of 28 kGy at a commercial facility (Schering Plough Animal Health Ltd, Upper Hutt, New Zealand).

Stock solutions of 10mg/ml of all chitosan were prepared in acetic acid (for crab chitosan 1% and for squid chitosan 2% was used). The stock solutions were diluted in sterile water to obtain concentrations of 0.63 mg/ml.

A stock solution of 10 mg/ml of Ampicillin (Merck, Germany) was prepared and was further diluted to 128 µg/ml in sterile water. Acetic acid 1% was diluted to 0.063% using sterile water.

3.4.5 Determination of minimum inhibitory concentration of *S. aureus* and *E. coli* O157:H7 using chitosan

96 well plates were prepared by dispensing 100 µl of sterile Mueller Hinton broth (MHB) to each of the wells. Into the first well of row A, B, C and D 100 µl of crab non-irradiated chitosan (0.625 mg/ml) was dispensed. Into the first well of row E and F 100 µl of Ampicillin solution (128 µg/ml) was dispensed. Into the first well of row G and H 100 µl acetic acid (0.063%) was dispensed. For all of the rows four, two-fold dilutions were performed. Each of the wells (except the wells in row C and D) were inoculated with 10 µl of the *E. coli* inoculum. Three wells containing MHB only were inoculated with *E.coli* and used as the growth control and three such wells were kept uninoculated as the sterility control.

On the same 96- well plate another set of wells were prepared using the same crab non-irradiated chitosan following the above described manner, but the wells were inoculated with 10 µl of the *S. aureus* inoculum while maintaining a sterility control and a growth as described above.

As mentioned above microdilution assays were performed for crab irradiated and squid pen (non-irradiated and irradiated) chitosans.

3.4.6 Chitosan treatment of meat samples inoculated with bacterial strains

Meat were first checked for the presence of *E. coli* O157:H7 and S. aureus ATCC 25923 by homogenizing the meat samples (25 g) in 225 ml of 0.1% peptone water (PW). Ten- fold dilutions were prepared (from the homogenized samples) and spread plates were prepared using Mannitol salt agar (Becton Dickinson, USA) and *E. coli*/coliform petrifilms (3M, USA) to

detect the presence of S. aureus and *E. coli* respectively. The samples negative for *E. coli* and *S. aureus* were used for the antimicrobial trials.

For the antimicrobial trials, meat samples were cut into 25 g portions and were inoculated separately with *E. coli* O157:H7 and *S. aureus* at inoculum levels ≅6 log each. The samples were stored at 20 °C for 30 min for cell attachment to occur. The meat samples were then separately treated (two samples for each treatment) with 50 ml (Ozturk & Sengun, 2019) of non-irradiated crab chitosan of concentration 1.25 mg/ml and 0.313 mg/ml, acetic acid (0.1%) concentrations of 0.125% and 0.031% and 0.1% peptone water (PW). The meat sample treated with 0.1% peptone was homogenized and ten-fold dilutions were prepared. Suitable dilutions were spread on *E. coli*/coliform petrifilms and Mannitol salt agar plates and incubated at 37^0C for 24h. After incubation the colonies formed were counted.

All samples were stored at 4^0C for 24h.

For the chitosan treatment trials, meat samples treated with PW and considered as the negative control. A positive control of meat treated with an antibiotic was not used.

3.4.7 Microbiological analysis

After 24 h, the meat samples from each treatment were transferred to separate empty stomacher bags (Nasco, USA). PW (225 ml) was introduced to each stomacher bag and the samples were homogenized using a stomacher (IUL, USA). The homogenates was then serially diluted in 9 mL of 0.1% PW. Inoculum of 0.1 ml was spread on duplicate of *E. coli*/coliform and duplicate plates of Mannitol salt agar for each sample and incubated at 37^0C for 24h for the enumeration of *E. coli* O157:H7 and *S. aureus* respectively.

3.5 Statistical analysis

Statistical significance between bacterial counts obtained for chitosan treated tripe (inoculated with *E. coli* and *S. aureus*) samples was analysed using analysis of variance (ANOVA) with a significance $P<0.05$.

Chapter 4 Results and Discussion

4.1 Microbiological analysis

The microbiological analyses were limited by availability of samples and it was difficult to obtain the lamb co-product samples in China. Thus, a lower sample size and exclusion of pizzle in the Chinese trials was unavoidable. Also, due to unavailability of media for *C. perfringens* identification during the research visit in China, this analysis was not conducted on the Chinese samples.

None of the sheep offal tested from either New Zealand or China was positive for *C. jejuni* (Table 4.1). Studies have shown that *Campylobacter* can potentially colonize intestinal mucosa of healthy sheep (Raji, Adekeye, Kwaga, & Bale, 2000 ; Butzler, 1985) and during slaughter contamination is highly likely. Sheep offal has been found to be susceptible to *Campylobacter* contamination in previous studies (Bolton et al.,1985; Little et al., 2008). A study from Great Britain where different offal samples (liver mostly, kidney and heart) were tested for the presence of *Campylobacter* reported that sheep (6-30%) offal samples had the highest *Campylobacter* incidence followed by cattle (5-10%) and pig (6%) (Bolton et al., 1985). In a study from UK, Little et al. (2008) reported a similar finding for sheep offal (liver, heart and kidney) that had a higher incidence (36.60%) of *Campylobacter* contamination than pork offal (liver, heart, kidney and tripe) (18.30%) and beef offal (liver, heart, kidney, oxtail and tripe) (12.24%). Though in the current study *Campylobacter* was absent in the liver samples (Table 4.1), sheep liver has been reported as a major reservoir of *Campylobacter* in past studies (Cornelius et al., 2005; Strachan et al., 2012). In a study conducted by Cornelius et al. (2005) in New Zealand, they reported *Campylobacter* incidences of 73.48% and 59.28% in sheep livers purchased from supermarkets and butcher's shops respectively. A study from Scotland also revealed a higher incidence of *Campylobacter* in livers of sheep (77.5%) (Strachan et al., 2012) . Sheep livers were also found to have a high *Campylobacter* incidence of 72.9% followed by the livers of pig (71.7%) and ox (54.2%) (Krameret al., 2000). Contamination by *Campylobacter* was found to take place during skinning and removal of the viscera, by transferring onto the carcass and the slaughter environment, through the spillage of intestinal fluid (Perko-Makela et al., 2009).The rupture of viscera is not uncommon during slaughter (Rosenquist, Sommer, Nielsen, & Christensen, 2006).

Table 4.1 presented below depicts the incidence of *C. jejuni*, *Salmonella*, *Listeria*, *C. perfringens* and *E.coli* in the sheep offal samples purchased from New Zealand and China.

Table 4.1 Prevalence of different pathogenic bacteria in sheep offal samples purchased from New Zealand and China

Country	Offal type	*Campylobacter jejuni* prevalence (%)	*Salmonella* spp. prevalence	*Listeria* spp. prevalence	*Clostridium perfringens prevalence*	*E. coli* prevalence
New Zealand	Kidney	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
	Heart	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
	Skirt	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
	Tripe	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
	Liver	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
	Tail	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
	Testes	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
	Pizzle	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)	0 (0/6)
China	Kidney	0 (0/4)	0 (0/5)	0 (0/5)		0 (0/3)
	Heart	0 (0/4)	0 (0/5)	0 (0/5)		-
	Skirt	0 (0/4)	0 (0/5)	0 (0/5)		0 (0/3)
	Tripe	0 (0/4)	0 (0/5)	0 (0/5)		0 (0/3)
	Liver	0 (0/4)	0 (0/5)	0 (0/5)		0 (0/3)
	Tail	-	0 (0/5)	0 (0/5)		-
	Testes	-	0 (0/5)	0 (0/5)		0 (0/3)

*Within parentheses are the number of samples positive as a fraction

Wieczorek, Denis, & Osek (2009) have shown that *C. jejuni* present on bovine hides (24.60%) contaminated 2.90% of the carcasses. This indicated that cross contamination of *Campylobacter* is likely to take place within a slaughter environment. During slaughter, washing and chilling are considered as the critical control points and are performed in every abattoir (Bolton et al., 2002). A positive effect of chilling carcasses towards reducing the *Campylobacter* incidence of meat have been reported in some studies (GRAU, 1988; Maramski, 2012). However, complete eradication of *Campylobacter* is impossible to achieve via chilling especially in the case of heavy contamination (Figueroa, Troncoso, López, Rivas, & Toro, 2009). Interestingly, it has been shown that *C. jejuni* has the ability to survive on disinfected surfaces by forming biofilms (Peyrat, Soumet, Maris, & Sanders, 2008). This is a mechanism used by the pathogen to combat stress and emphasises the need to ensure effective disinfectants are used in the food industry. These reasons explain the possible causes for the *Campylobacter* positive findings reported in sheep offal in previous work.

The absence of *C. jejuni* in the New Zealand sheep offal (Table 4.1) maybe due to the effective implementation of the *Campylobacter* risk management strategy 2017-2021 by the Ministry for Primary Industries (MPI). Reducing the Campylobacteriosis incidence by 10% per 100,000 per head of population by the end of 2020 was among the prime goals of the *Campylobacter* risk management strategy. MPI is the government body which ensures food safety in New Zealand. Within the time period from 2007-2012 Campylobacteriosis was reduced by more than 50% as a result of the Campylobacter Risk Management Strategy (MPI, 2020). Overall, the samples being frozen may also have contributed towards the absence of *C. jejuni* in the samples. In a study where fresh, chilled and frozen poultry from a commercial processing plant were examined in the fresh, chilled and frozen states, the incidence of *C. jejuni* were found to be 93.30, 53.30 and 36.70% respectively (Maziero et al., 2010). These results indicate that freezing reduces *C. jejuni* incidence. This maybe a result of cell death which occurs due to oxidative stress, ice nucleation and dehydration (Maziero et al., 2010).

None of the sheep offal tested from either New Zealand or China was positive for *Salmonella* (Table 4.1). However, Salmonella has been isolated from meat offal in previous studies (Im et al., 2016; Little et al., 2008). Im et al.(2016) in a study from Korea reported a higher *Salmonella* incidence (23.81%) in sheep offal (Heart, liver, lung, tripe, small intestine and large intestine) compared to the same offal obtained from cattle (7.10%). A much lesser *Salmonella* incidence (3.11%) was reported in lamb offal (liver, heart and kidney) by Little et al. (2008). As *Salmonella* contamination mainly occurs via cross contamination during slaughter and evisceration, the varying degrees of prevalence serves as an indication of the hygienic practices conducted during the meat slaughter and meat processing procedures. Since the processing of meat and offal are separately performed, if efficient hygienic practices are maintained, then cross contamination could be avoided. Absence of *Salmonella* has been previously reported in beef meat, sheep meat and beef offal by Cohen et al. (2006). In previous studies Salmonella incidence had been detected in New Zealand retail meat such as pork (0.1%), beef (0.4%) and lamb (1.3%) (Duffy et al., 2001; Sofos et al., 1999; Wong et al., 2007). In China, retail samples of chicken (54%), pork (31%), beef (17%) and lamb (20%) samples were found to be positive for Salmonella (Yang et al., 2010). Proper handling of the offal samples may have led to the absence of *Salmonella*. Furthermore, frozen meat is found to have a lower *Salmonella* incidence than raw meat. Sinell et al., (1984) showed that frozen livers purchased from the Central Wholesale Meat Market in Berlin had a *Salmonella* incidence of 29.3% compared to the incidence in fresh liver (72%). Since the samples used were frozen, this may have contributed to the absence of *Salmonella* in the offal.

There was no Listeria spp. detected in any of the offal samples obtained from New Zealand or China (Table 4.1). Slaughtered animals are known sources for foodborne pathogens (Kuan et al., 2013). A major cause for *Listeria* outbreaks is inefficient hygiene and control of contamination in food processing plants (Todd & Notermans, 2011). The absence of *Listeria* in the samples analysed in the present study indicate that efficient HACCP practices had been carried out from the point of slaughter till storage of the offal products. Similar findings where no *Listeria* was detected in red meat has been reported in previous studies (Mashak et al., 2015; Cohen et al., 2006).

Mashak et al. (2015) reported the absence of *Listeria* in frozen sheep meat, but 8.33% of fresh meat was found to be contaminated by *Listeria*. Cohen et al. (2006) also reported the absence of *Listeria* in chilled samples of beef and sheep meat and beef offal (heart and liver) purchased from butcheries, supermarkets and slaughterhouses. It had also been reported in a study from Iran that the *Listeria* incidence in fresh meat is much greater than in frozen meat (Mashak et al., 2015). *Listeria* is a psychrotrophic bacterium and is capable of proliferating at temperatures as low as 4^0C. This could explain the higher numbers observed at chilled temperatures. The degree of contamination may also depend on the initial load of *Listeria* present on the meat prior to cold storage. However, Kuan et al. (2013) reported the presence of 25, 50, 22, 46.67 and 0% *Listeria* in fresh chilled samples of beef liver, lung, intestine, tripe and spleen (purchased from wet markets), respectively. In a separate study, Kuan et al. (2013) also reported the presence of *L. monocytogenes* in fresh chilled samples of chicken offal purchased from wet markets and hypermarkets. The incidence of contamination by *L. monocytogenes* in the chicken liver, heart and gizzard were 25, 20 and 33%.

Pork loins obtained from a federal inspection type (FIT) slaughterhouse had less *Listeria* prevalence (15.7%) than in non-FIT slaughterhouse (25.7%) (Alejandro et al., 2019). The FIT slaughterhouse is strictly regulated by International Standards of Quality and Hygiene compared to the non-FIT slaughterhouse. The difference in results is a clear indication of the impact strict regulations have upon *Listeria* control in meat. However, even under the control of such regulations, complete eradication of *Listeria* is difficult to achieve. Complete eradication of *Listeria* is challenging due its ability to adhere to surfaces of the food processing environment and form biofilms (Alejandro et al., 2019).

In a Chinese study, meta-analysis was carried out that included literature from 2000-2016 to investigate the prevalence of L. *monocytogenes* in meat products in China (Liu et al., 2020). It

was revealed that pooled prevalence of *L. monocytogenes* in fresh pork, beef, mutton, poultry and frozen raw meats were found to be 11.4, 9.1, 5.4, 7.2 and 7.2%, respectively. Studies performed before 2010 had a higher *L. monocytogenes* incidence (13.3%) in raw meat compared to the studies performed after 2010 (9.8%). The food safety is at a much improved state at present and the present results highlight the continued progress made in China to control foodborne pathogens. However, as red meat had been shown to be a reservoir for *L. monocytogenes* it is important to take advanced measures to prevent its incidence on meat. In another Chinese review where the prevalence of *Listeria* in different food products was investigated (included research published from 2008-2016), it was revealed that the meat (includes pork, beef, and mutton) and poultry products had the highest Listeria prevalence (8.91%) compared to aquatic animals, salads, rice and flour products, vegetables and dairy (Weiwei et al., 2018). These findings emphasised the significance of red meat products as a source of *L. monocytogenes*. Rivas et al. (2017) reported that New Zealand RTE red meats had a 6.4% *L. monocytogenes* prevalence. Previous New Zealand studies had also reported *L. monocytogenes* prevalence of 4.3% in unpackaged ham (Cornelius et al., 2008) and 1% in vacuum packaged meat (Wong et al., 2005). These findings indicate the possibility of *L. monocytogenes* occurrence in meat. Therefore, and there is a need to develop and implement new pathogen risk management strategies to maintain New Zealand offal at a high quality.

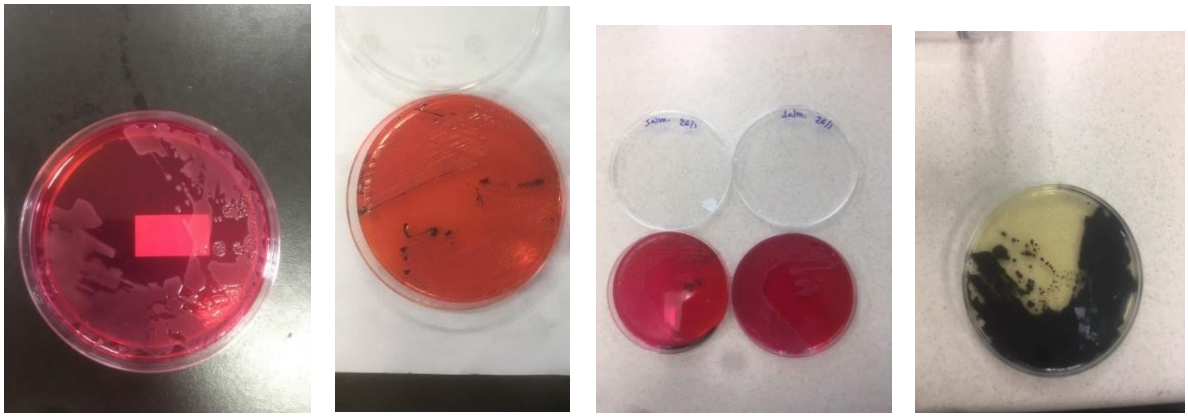
C. perfringens was not detected in any of the analysed New Zealand sheep offal samples (Table 4.1). At present, in New Zealand the meat processing plants use a spray containing peroxyacetic acid in the plant between shifts and after cleaning to kill clostridial spores (Brightwell et al., 2018). The absence of *C. perfringens* may be a positive outcome of this strategy. The present study's findings contradict results reported by Im et al. (2015) where he found *C. perfringens* present in 11.10% of pig offal samples and 7.10% cattle offal samples. Lee and Lee (2016) reported *L. perfringens* prevalence of 3.70% in the small intestine of pig and 1.80% in the cattle omasum. These results are understandable as *Clostridium* spp. is known to be present in the ruminant gastrointestinal tract (Cohen et al., 2006). These results suggested the need for a more refined HACCP system for offal processing in Korea. In a study from Morocco where the microbiological profiles of lamb meat, cattle meat and cattle offal were investigated, out of all the meat analysed 4.5% of the samples exceeded the tolerable *Clostridium perfringens* limit (1.3 log CFU/g) set by the Moroccan food safety regulations (Cohen et al., 2006).

The occurrence of psychrotolerant *Clostridium* spp. in New Zealand meat leading to "blown pack " spoilage was reported in some studies (Brightwell et al., 2018; Broda et al., 2002; Broda

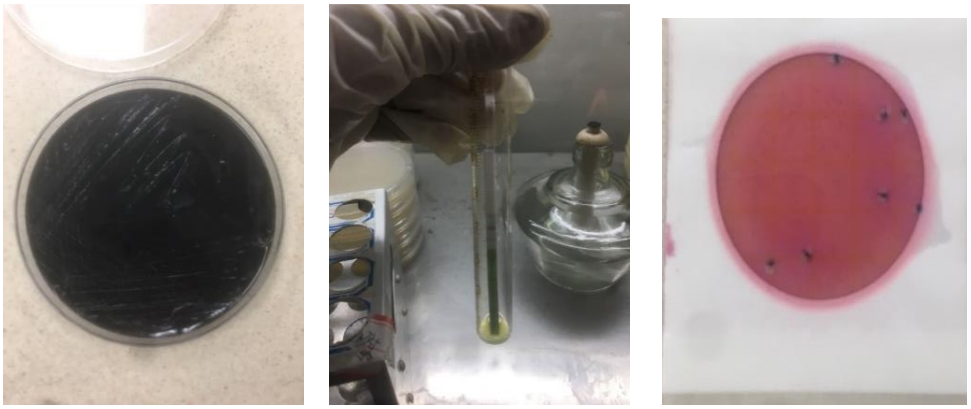
et al., 2003). In a recent study, 19.35% of New Zealand lamb meat juice samples obtained from meat exported to Switzerland were positive for *Clostridium* spp. (Wambu et al., 2020). This finding agrees with results from previous New Zealand studies where psychrophilic *Clostridium* was isolated from lamb meat (Brightwell et al., 2018; Broda et al., 2002; Broda et al., 2003). *Clostridium difficile* had been isolated from uncooked meat sausage containing pork in New Zealand (Rivas et al., 2019). However, the isolates PCR ribotype was found to be RT045, which was not reported in any New Zealand human cases (Rivas et al., 2019).

Furthermore, in a New Zealand study where psychrotolerant *Clostridium* isolates obtained from beef, lamb and deer were studied, 13 *Clostridium* isolates responsible for spoilage of vacuum packaged chilled red meat were identified (Brightwell et al., 2018).

Figure 4.1 depicts all of the positive controls used in microbiological analysis of the present study.



(A) **(B)** **(C)** **(D)**



(E) **(F)** **(G)**

Figure 4.1 Positive controls of *Salmonella* CMCC 50094 on BGM (A) and XLD (B) agar *S. typhi* B8 on XLD (left) and BGM agar (C), *C. perfringens* NZRM 20 (D), *C. jejuni* ATCC 2397 (E), *L. monocytogenes* ATCC 19111 (F) and *E. coli* NZRM 916.

Table 4.2 presents the results obtained for the coliform counts of the sheep offal purchased from New Zealand and China. Due to the shortage of samples mentioned earlier the analysis was not conducted for the heart, tail and pizzle samples purchased from China.
Table 4.3 presents the results obtained for the APCs of the sheep offal purchased from New Zealand and China.

Table 4.2 Coliform count of sheep organs purchased from New Zealand and China

Organ	Coliform count (log CFU/g)	
	New Zealand	China
Testes	0	4.670 ± 0.955
Skirt	0	5.098 ± 0.598
Liver	0	5.007 ± 1.016
Tripe	0	4.769 ± 0.52
Kidney	0	7.117 ± 0.162
Heart	0	-
Tail	0	-
Pizzle	0	-

Table 4.3 Aerobic plate count of sheep organs purchased from New Zealand and China

Organ	Aerobic plate count (log CFU/g)	
	New Zealand	China
Testes	1.845 ± 0.578	6.274 ± 0.250
Skirt	1.651 ± 0.531	6.038 ± 1.531
Liver	1.412 ± 0.278	6.357 ± 0.723
Tripe	0	5.704 ± 0.917
Kidney	1.605 ± 0.512	7.562 ± 0.581
Heart	1.533 ±0.967	7.406 ± 0.556
Tail	2.160 ± 0.181	7.413 ± 0.449
Pizzle	2.349 ± 0.461	7.438 ± 1.114

E. coli (Table 4.1) was not detected in any of the frozen sheep offal samples purchased from New Zealand and China. However, *E. coli* had been present in various Chinese meat according to previous studies (Li et al. 2019; Zhang et al., 2016). Li et al. (2019) observed a significant difference in *E. coli* counts in frozen poultry compared to fresh (1.60 log CFU/g) and chilled

(1.81 log CFU/g) poultry. Furthermore, a lower number of frozen poultry samples were contaminated by *E. coli* (30.31%) compared to the fresh (53.92%) and chilled poultry samples (60.66%). These results indicate that *E. coli* growth is suppressed at freezing temperatures. In a study where *E. coli* incidence in retail meats obtained from Sichuan province (China) was examined by Zhang et al. (2016) chicken was found to have a higher *E. coli* incidence (84.8%) followed by pork (79.3%) and beef (66.7%). Investigating fresh raw meat samples purchased from supermarkets and open markets in South China revealed a higher incidence of *E. coli* O157 in beef compared to pork (6.90 %), chicken (3.28 %) and duck (2.54 %). However, *E. coli* O157 was not present in any of the investigated mutton samples (Zhang et al., 2016).

In the New Zealand sheep offal samples none of the samples showed the presence of coliforms (Table 4.2). As coliform presence in food is considered as an indication of poor food handling practices, absence of coliforms indicate that proper food handling practices under sterile conditions have been carried out during the preparation of New Zealand meat samples. In the Chinese sheep offal samples a higher incidence of coliforms was observed in the kidney (7.117 ± 0.162 log CFU/g) followed by skirt (5.098 ± 0.598 log CFU/g), liver (5.007 ± 1.016 log CFU/g), tripe (4.769 ± 0.520) and testes (4.670 ± 0.955 log CFU/g) (Table 4.2). These results indicate that the Chinese meat samples were handled under poor sanitary conditions. Lower coliform counts for offal meat had been reported in previous studies (Ibrahim et al., 2013; Cohen et al., 2006). Cattle liver, kidney and lung purchased from a slaughter house in Egypt were found to have coliform counts of 4.53, 4.34 and 4.51 log CFU/g respectively (Ibrahim et al., 2013). Cohen et al. (2006) reported coliform counts of 2.50, 2.80 and 2.3 log CFU/g in chilled fresh samples of beef, lamb and beef offal (heart and liver) respectively. Bensink et al. (2002) had reported that the use of wet dumping (using 20-30 L of water to empty paunches) on tripe (scalded with water at 80^0C) and freezing could results in coliform counts less than 4 log/cm^2. The high coliform count observed in the Chinese tripe samples may have been due to inefficient cleaning. Inadequate feed withdrawal result in intestines partially filled with faeces and feed. This would lead to offal contamination during the slaughter process (Rasschaert et al., 2020). Accumulation of faeces and feed in the intestines may have led to the high coliform counts the Chinese offal samples. A similar finding was reported by Liu et al. (2019) in a study where the quality of chilled pork tenderloins purchased from online meat companies in China was investigated it was found that at end point temperature <4 °C (0.83 ± 0.40 log MPN/g) coliform counts were significantly lower ($p<0.05$) than at 4–10 °C (1.22 ± 0.70 log MPN/g) and >10 °C (1.95 ± 0.36 log MPN/g) (Liu et al., 2019). Furthermore, at temperatures <4 °C, 4–10 °C

and >10 °C the unqualified rates of the examined pork samples due to coliform presence were found to be 0, 19.0 and 60%, respectively. MPN stands for most probable number which provides an estimation of population density without counting single colonies or cells (Alexander, 1983).

China is the largest producer and consumer of antimicrobial compounds in the world (Zhang et al., 2016). Quaternary ammonium compounds (QACs) (0.02-0.1%) are used in China to meet the disinfection requirements of slaughterhouses and meat production facilities. These compounds are known to inhibit the growth of *E. coli*. However, the widespread use of QACs may give rise to resistance to antibiotics in bacteria (Zhang et al., 2016). Furthermore, antibiotics such as kanamycin, ampicillin, tetracycline, streptomycin and sulfamethoxazole are used in meat production facilities as disinfectants and they are also known to contribute to antibiotic resistance in bacteria (Zhang et al., 2016). The absence of *E. coli* in the Chinese meat samples may have been due to the use of antibiotics. However, the presence of coliforms may have been an indication of antibacterial resistance developed by them.

APC is another indicator utilized to investigate microbial contamination of food. As the APC guidelines for New Zealand and Chinese meat was not available the ICMSF criteria was used to evaluate the APC counts in sheep offal. According to ICMSF criteria, meat offal samples which exceed 6 log CFU/g are considered to be unsuitable for consumption (Im et al., 2016). All of the investigated New Zealand offal had mean APC values which meet the ICMSF criteria (Table 4.3). These APC values are lower than APC counts reported in previous studies (Cohen et al., 2006; Li et al., 2019). In a study from Morocco Cohen et al. (2006) reported that the mean APC counts obtained for beef, lamb and beef liver (fresh and chilled) were 6.5l, 6.2l, and 5.0l log CFU/g respectively. In contrast to the New Zealand offal, APC values of majority of the Chinese offal samples (except for tripe) failed to meet the ICMSF criteria (Table 4.3).

In a study where poultry samples (duck and chicken) obtained from different provinces in China were investigated for the presence of aerobic plate counts, it was found that a significantly lower count was observed in frozen poultry (and 4.85 log CFU/g) compared to the fresh (5.53 log CFU/g) and chilled (5.43 log CFU/g) poultry (Li et al., 2019). This finding clearly indicate that frozen samples have a lower incidence aerobic bacteria. The frozen state of the samples alongside effective meat handling procedures may have led to the lower APC values in the New Zealand meat.

In a previous study in which microbial levels of meat purchased from retail shops were examined it was revealed that the coliform count was positively correlated to APC (Kim & Yim, 2016). Though the correlation between coliform count and APC was not investigated in the present the trend is clearly visible in the results. For instance, the Chinese kidney samples with the highest coliform count (7.12 ± 0.16 log CFU/g) also had a similarly high APC (7.56 ± 0.58 log CFU/g).

In the present study, frozen offal samples were used instead of fresh samples. A reason for this was the difficulty to source fresh sheep offal from New Zealand due to the low market demand. In New Zealand, sheep offal are mainly exported frozen. For the China trials, the offal samples were sourced from the Northern region of China, while the trials were conducted in the Southern region of China. Therefore, frozen samples were the only option available, which reflect the actual situation for offal market.

Overall, in the New Zealand sheep offal the investigated foodborne pathogens (*E. coli*, *Campylobacter jejuni*, Salmonella, *Clostridium perfringens*, and *Listeria monocytogenes)* were not detected. This may be due to following the Microbiological limit Standard 1.6.1 implemented by Food Standards Australia New Zealand (FSANZ), to ensure food with unsafe levels of microbes are not introduced to consumers (FSANZ, 2019). In addition to determining the safety level of food the standard also helps to identify accurate functioning of processing steps.

Similar to the New Zealand samples none of the investigated pathogens were identified Chinese sheep offal. This may have been a result of food safety being made a national priority by the Chinese government since 2009. In the year 2013 certain amendments were made to the Food Safety Law 2009 with an emphasis on risk-based standards (Dong et al., 2015). The absence of the investigated foodborne pathogens in Chinese sheep offal may have also been a result of the attempts of the Chinese government to ensure food safety.

APC values of all New Zealand offal met the ICMSF criteria. However, majority of the Chinese offal samples (except for tripe) failed to meet the ICMSF criteria. This suggests that more attention needs to be payed towards the implementation of HACCP guidelines in the meat handling process. There were no coliforms detected in the New Zealand samples, but in the Chinese samples had a high incidence of coliforms in the kidney, skirt, liver, tripe and testes (Table 4.2). These results indicate that the Chinese meat samples were handled under poor sanitary conditions and more attention needs to be payed towards this.

4.2 Metagenomic analysis of sheep offal

Figures 4.2 and 4.3 show the percent of community abundance of different phyla present in the meat offal samples purchased from China and New Zealand, respectively. Figures 4.4 and 4.5 show the percent of community abundance of different genera present in the meat offal samples purchased from China and New Zealand, respectively. Figures 4.6 and 4.7 show the percent of community abundance levels of different species present in the meat offal samples purchased from China and New Zealand, respectively.

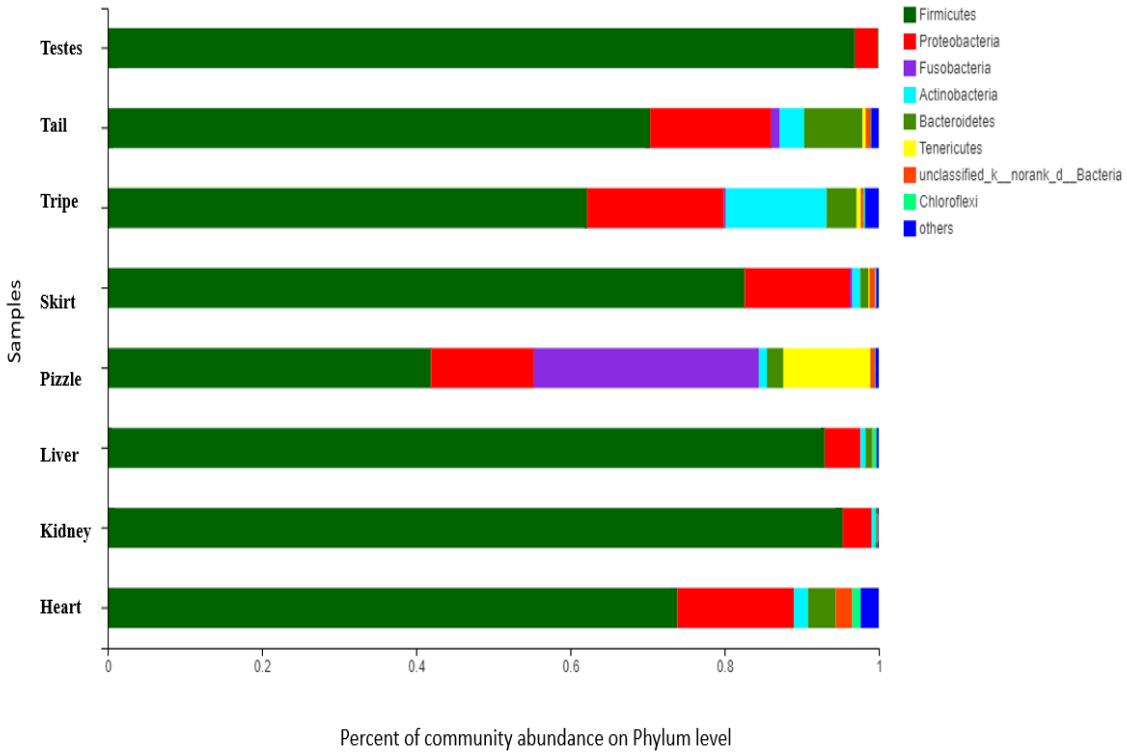


Figure 4.2 Percent of community abundance at Phylum level for sheep offal samples purchased from China.

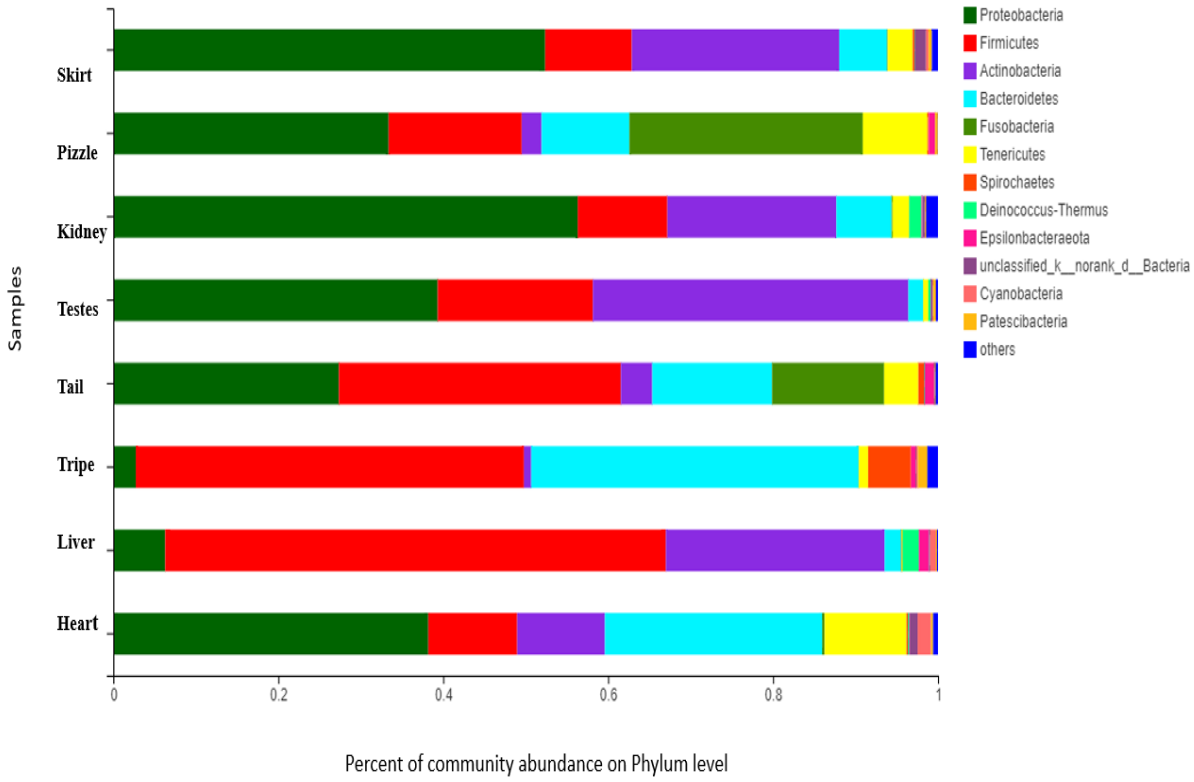


Figure 4.3 Percent of community abundance at Phylum level for sheep offal samples purchased from New Zealand.

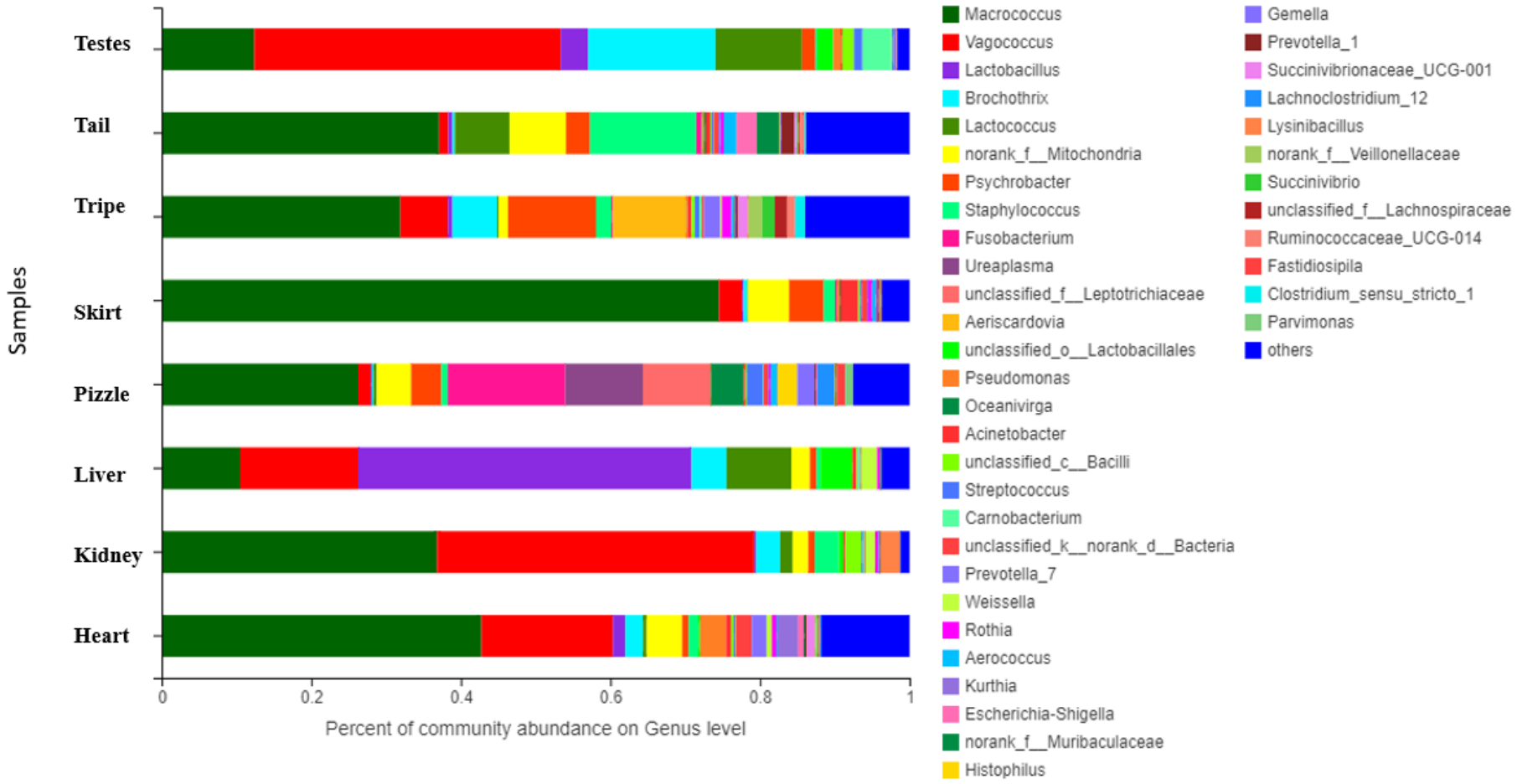


Figure 4.4 Percent of community abundance at Genus level for sheep offal samples purchased from China.

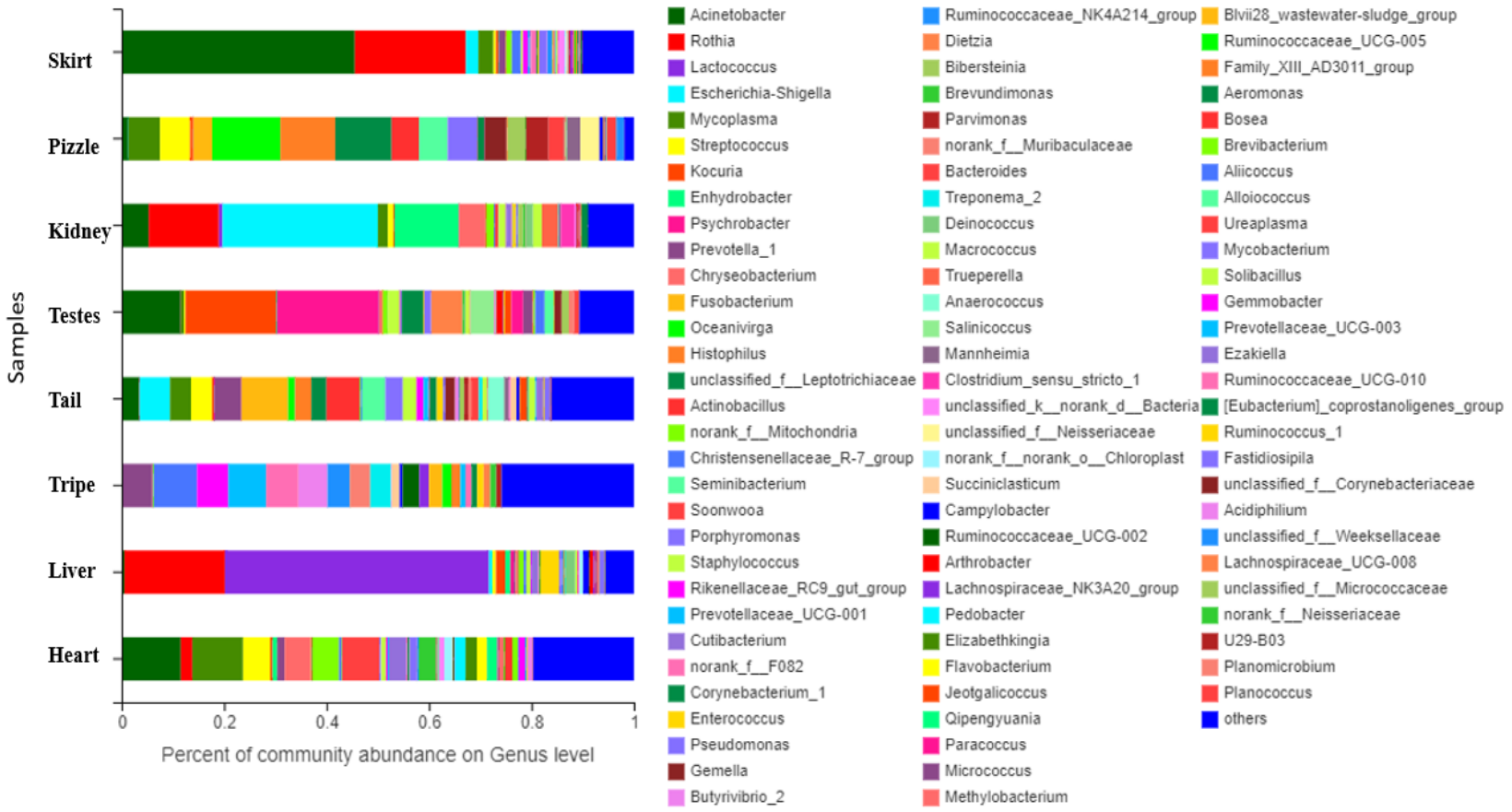


Figure 4.5 Percent of community abundance at Genus level for sheep offal samples purchased from New Zealand.

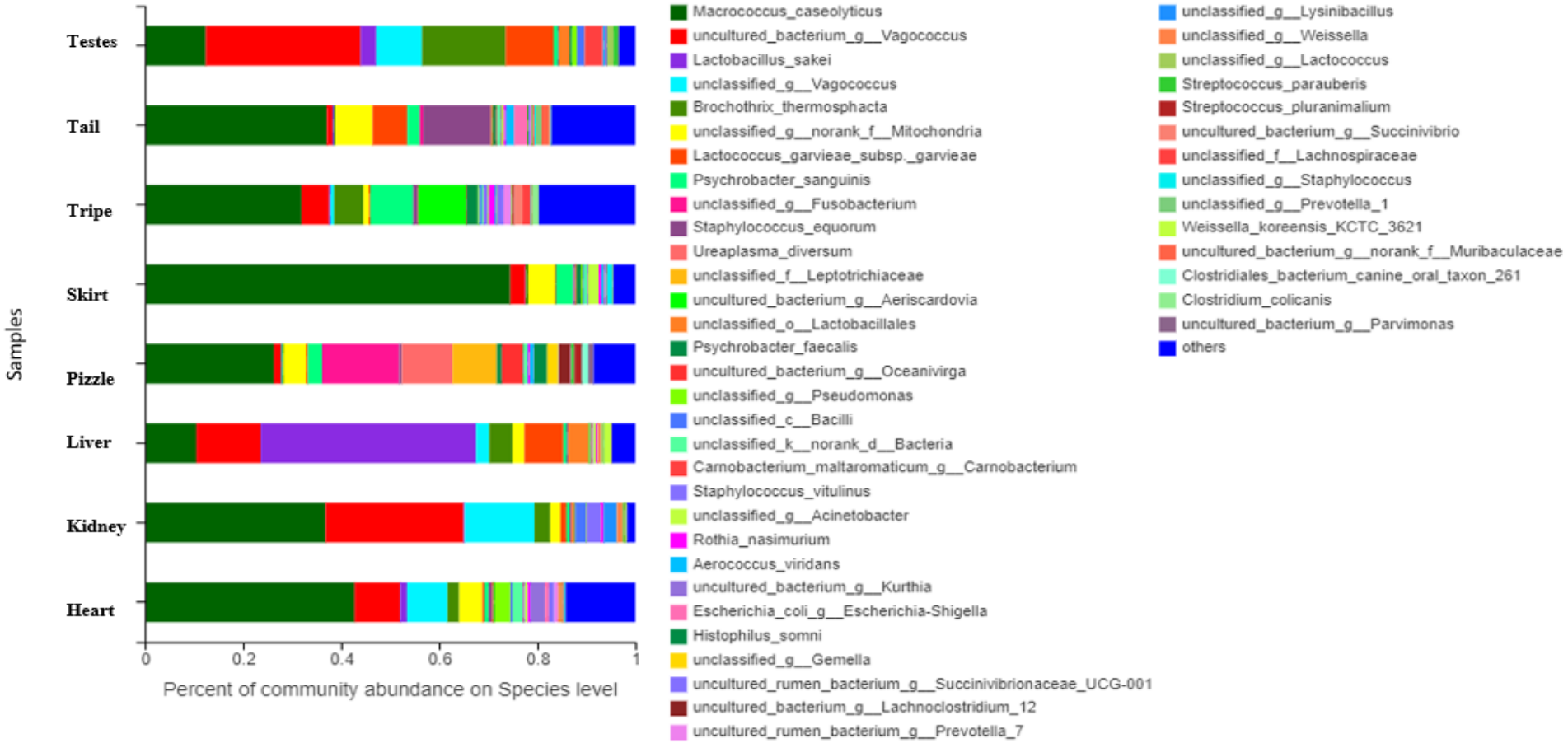


Figure 4.6 Percent of community abundance at Species level for sheep offal samples purchased from China.

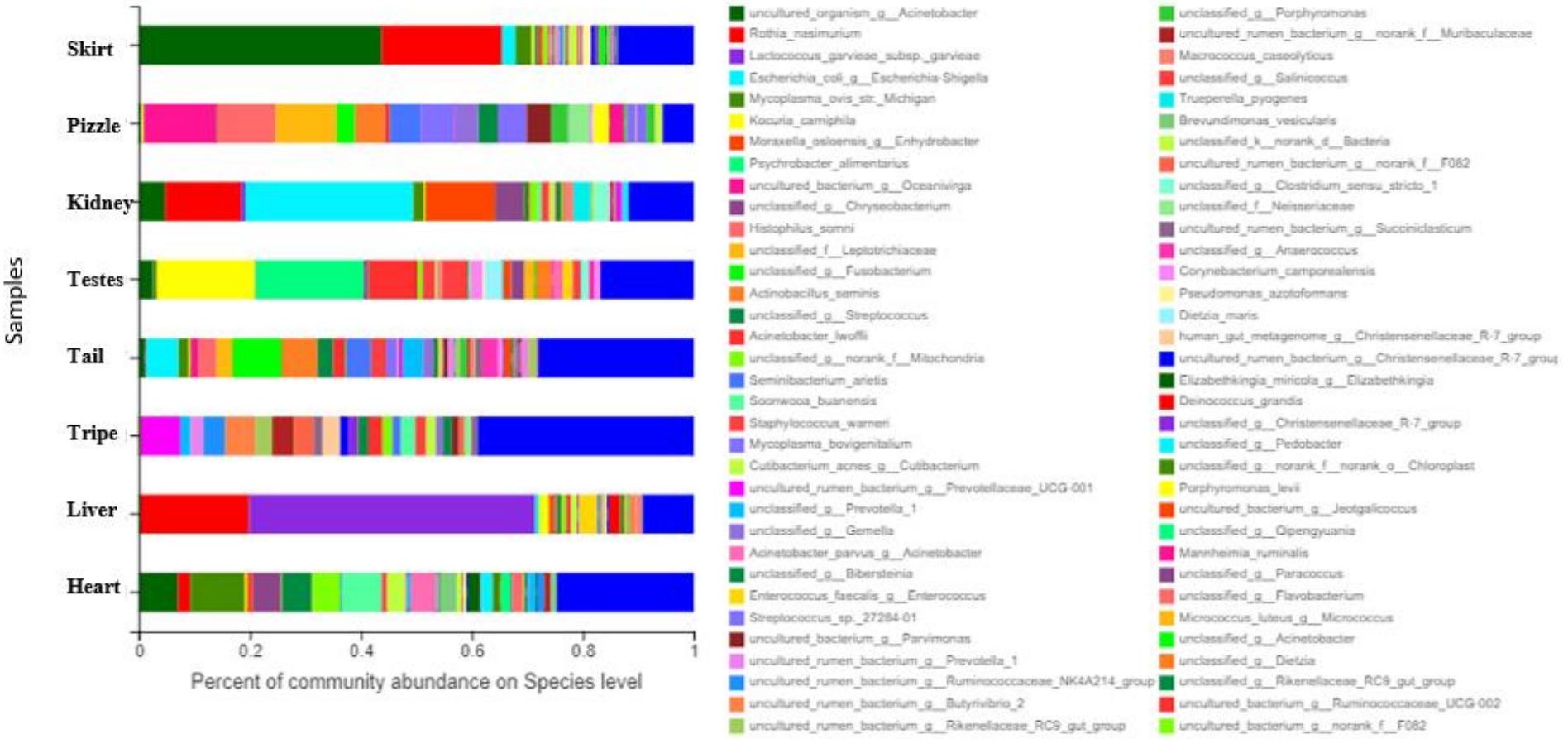


Figure 4.7 Percent of community abundance at Species level for sheep offal samples purchased from New Zealand.

In the Chinese samples, Firmicutes were the dominant phylum in all samples, followed by Proteobacteria. However, in the pizzle samples there were more Fusobacteria than Proteobacteria. Among all samples, a significant presence of Tenericutes and Fusobacteria were present mainly in pizzle (Figure 4.2).

In New Zealand offal, Proteobacteria was found to be the most dominant phylum in skirt, pizzle, kidney, testes and heart samples as opposed to the Chinese samples. Fermicutes were the most dominant phylum in the liver and tripe samples similar to the Chinese offal. Yang et al. (2016) reported that the predominant phyla in beef during various stages in production chain were Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. However, the proportions of Actinobacteria, Firmicutes, and Bacteroidetes were changed from one stage to another (Figure 4.2).

Proteobacteria is the largest bacterial phylum consisting of Gram-negative members that are obligate or facultative anaerobic. It is speculated that Proteobacteria lowers the redox potential in the gut facilitating the colonization of strict anaerobes needed for healthy gut function (Moon et al., 2018). Proteobacteria are capable of growing on a range of organic compounds such as lipids, protein and carbohydrates (Moon et al., 2018). Gamma-proteobacteria is the largest and most diverse class of Proteobateria. *Enterobacteriaceae* and *Pseudomonadaceae* are two significant families belonging to Gamma-proteobacteria, which include human pathogens (Deak, 2010). *Salmonella*, *Shigella*, *Escherichia* and *Yersinia* are some of the pathogenic enterobacteria while *P. aeruginosa* is a well-known food spoilage bacteria (Deak, 2010).

Bacteroidetes are also a diverse phylum that play a major ecological role in the carbon cycle due to the ability to degrade compounds of high molecular weights. This phylum do not contain notable food pathogens but can exist as food contaminants. In warm-blooded animals, Bacteroidetes are present in the gastrointestinal tract, and some may be present as opportunistic pathogens. Firmicutes is a bacterial phylum consisting of genera such as *Listeria*, *Clostridium* and *Staphylococcus,* which consist of the major foodborne pathogens.

It was shown in a study by Liu et al., (2019) that diet plays a major role in determining the relative abundance of different phyla of rumen microbial community. In Tibetan sheep that were fed high-concentrate diets, a high proportion of *Bacteroidetes* (55.02%) were found in rumen fluid compared to *Proteobacteria* (22.10%). *Bacteroidetes* are known to be capable of degrading non-fibrous carbohydrates and non-fibrous polysaccharides (Liu et al., 2019).

Therefore, the high abundance of *Bacteroidetes* may have been due to the presence of a higher proportion of non-fibrous carbohydrates and polysaccharides in the high concentrate feed (Liu et al., 2019). The difference in community abundance between the Chinese and New Zealand samples may have been due to the difference in diets. Though the results of the present study provide an overview of the different microbial populations present in the samples to investigate the presence of pathogens a species level analysis is required.

At the species level most of the foodborne pathogens (except *E. coli*) were not detected through conventional culture methods in the New Zealand samples (Figure 4.7). A high amount of *E. coli* was detected in kidneys (30%) while < 6% was detected in tail, skirt and liver samples. However, *E. coli* were not detected from any of the New Zealand offal during the microbiological analysis performed using the 3M petrifilms. This observation suggests that viable *E. coli* was absent in the offal samples, but non-viable cells may have been present on the samples.

The results of the metagenomic analysis of the New Zealand offal revealed the presence of few other potential foodborne pathogens at Phylum or genus level (Figure 4.5). *Acinetobacter*, *Pseudomonas*, *Brochothrix*, *Psychrobacter*, *Staphylococcus* and lactic acid bacteria, are the commonest genera present on fresh meat (Odeyemi et al., 2020). Furthermore, these bacteria are capable of causing meat spoilage during cold storage (Odeyemi et al., 2020). A high abundance level of *Acinetobacter* (44%) was detected in skirt samples and relatively low levels (< 7%) were detected in kidneys, testes, tail and heart. *Acinetobacter baumanni* (causes infections in wounds and urinary tract) has previously been isolated from sheep meat (41.12%) (Askari et al., 2019). These findings indicate that further investigations are required to confirm if the detected phyla and genera in the metagenomics analysis are capable of growing using traditional microbiological methods. *Lactococcus* was found to be present at a very high level in the New Zealand sheep liver (52%). The species *Lactococcus garviae*, has been identified as an emerging opportunistic pathogen in humans (Ricci et al., 2013). Previously, the organisms belonging to the genus *Lactococcus* were known to have low virulence causing human infections recent, however, recent reports have indicated an association between consumption of raw fish and *L. garviae* related infections (Choksi & Dadani, 2017). This suggests the need to monitor the occurrence of *L. garviae* in New Zealand sheep liver. *Psychrobacter* was present only in the testes samples.

Chinese sheep liver showed a high abundance of *Lactobacillus* (45%). *Lactobacillus sakei* was the main identified species (Figure 4.6). In contrast to the New Zealand offal, the Chinese offal showed the presence of psychrotrophic *Brochothrix*. *Brochothrix thermosphacta* is an important organism responsible for the spoilage of refrigerated meat (Hernandez-Macedo et al., 2011). Its aerobic metabolism results in lactic acid and ethanol leading to off-flavours in the meat (Hernandez-Macedo et al., 2011). The testes samples had the highest abundance levels (17%) while the abundances in the tripe, liver, kidney and heart was < 6% (Figure 4.4). As opposed to the New Zealand samples, in all of the Chinese offal types *Psychrobacter* was present. The highest abundance was in the tripe (15%), while the rest of the offal had a low abundance (< 5%) (Figure 4.4). *Staphylococcus* was detected the most in the tail (14%) and at a lower level (> 5%) in most of the offal excluding testes. *S. equorum*, a common microbe used in European fermented meat, was the species identified in the tail (Figure 4.6). Food poisoning and pathogenicity related to *S. equorum* have not been reported (Jeong et al., 2017).

At the species level, all the Chinese offal contained *Macrococcus caseolyticus*. Species belonging to the genus *Macrococcus* are commonly isolated from meat and milk products. These bacteria (Gram-positive, catalase-positive) share a close relationship with staphylococci. However, in contrast to staphylococci, micrococci are not considered as pathogens. Yet, *Macrococcus caseolyticus* and *Macrococcus canis* have recently been identified as organisms involved in veterinary infections (MacFadyen et al., 2018). This finding indicates the need to further investigate the presence of this species in meat and develop strategies to minimise its occurrence. Species of the genus *Vagococcus* were also found abundant in the Chinese sheep offal. Species belonging to this genus such as *Vagococcus penaei* are known to cause spoilage in cooked shrimp (Jaffres et al., 2010). It has been reported by Lauritsen et al. (2019) that in whole broiler meat (packed in 80%O_2/20%CO_2 modified atmosphere) *Vagococcus* dominate later shelf-life microbiota. Therefore, further investigation on the presence of *Vagococcus* in Chinese sheep offal would be recommended.

In general, a higher diversity at the Species level was exhibited by the New Zealand sheep offal in comparison to the Chinese samples. The reason for the higher level of diversity cannot be established due to the availability of limited research published on metagenomic analysis of sheep offal.

Overall, at the phylum level the Chinese offal samples were dominated by Fermicutes. Proteobacteria, Tenericutes and Fusobacteria were the other major phyla detected in the samples. As opposed to the Chinese samples the New Zealand samples were dominated by Proteobacteria in some organs while some organs were dominated by Fermicutes.

Actinobacteria and Bacteroidetes were the other commonly detected phyla in the New Zealand samples.

Contrary to the microbiological analysis *E. coli* was detected in some of the New Zealand samples suggesting the presence of non-viable *E. coli* cells on the meat surface. At the genus level a few meat spoilage causing genenra such as *Acinetobacter*, *Pseudomonas*, *Brochothrix*, *Psychrobacter*, *Staphylococcus* and lactic acid bacteria were detected in the New Zealand samples suggesting the need for extensive studies on the different microbial populations present on frozen meat offal. In the Chinese samples the genera *Brochothrix*, *Macrococcus*, *Vagococcus* and lactic acid bacteria were present. As these genera include species which could result in meat spoilage and possibly human infections it is important to further study the different microbial populations present on frozen Chinese sheep offal.

4.3 Analysis of mycotoxins in sheep offal

Aflatoxins are the most important mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*. The presence of residues of these mycotoxins in animal tissues is a major concern to public health (Hassan et al., 2014) All of the New Zealand offal types examined were positive for AFB_1 (Table 4.4). The highest prevalence was observed in skirt samples (13.77±7.55 µg/kg) and the lowest was in liver (0.88±0.76 µg/kg). A maximum limit for total aflatoxins in red meat has not been established by The Australia New Zealand Food standards code. All of the aflatoxin levels present in the New Zealand offal samples were less than 15µg/kg, which is the established maximum limit for total aflatoxins in peanuts and tree nuts by The Australia New Zealand Food standards code (The New Zealand Mycotoxin Surveillance Program 06-14 Report series, 2014)

In a study from Egypt where sheep carcasses were investigated for the presence of mycotoxins AFB_1 levels of 41.69±2.53 , 32.80±2.14 and 26.85,1.79 µg/kg were reported from three individual abattoirs (Hassan et al., 2014). AFB_1 excretes via the urinary pathway and this would result in a higher AFB_1 occurrence in the kidney (Li, Xing, Zhang, Wang, & Zheng, 2018). In a separate study from Egypt, beef liver and kidney were found to have 0-20 and 3.8-24 µg/kg of AFB_1 respectively (Hassan et al., 2014). A higher concentration of AFB_1 was reported in the kidneys (6.55±6.09 µg/kg) than in the liver. In the New Zealand samples a similar trend was observed as the kidneys had a higher concentration of AFB_1 compared to liver (0.88±0.76 µg/kg).

AFB_1 was present in tripe (0.29 µg/kg) and liver (0.83 µg/kg) samples purchased from China. However, from all the samples analysed only one sample from each offal type had AFB_1 present. AFB_1 was not present in any of the other offal types. In the liver, the microsomal cytochromes P450 convert AFB_1 to less toxic M_1. This biotransformation at the hepatic level maybe the reason for higher incidence of AFB_1 in the liver (Völkel, Schröer-Merker, & Czerny, 2011). National Food Safety Standard for Maximum Levels of Mycotoxins in Foods released by the National Health and Family Planning Commission and the China Food and Drug Administration does not mention a maximum limit for AFB_1 in meat products (National Food Safety Standard for Maximum Levels of Mycotoxins in Foods, 2017).

T-2 toxin is a tricothecene mycotoxin of type A produced by different Fusarium species. Food and feed contamination by the T-2 toxin is a global food safety issue (Zeng et al., 2019). Incidence of T-2 toxin was observed in all of the kidney, 40% of heart and 20% of pizzle samples of New Zealand. T-2 toxin was absent in rest of the New Zealand offal types. In the

Chinese samples,T-2 toxin was present only in skirt samples (0.03±0.01 µg/kg). In comparison to monogastric, ruminants are known to show resistance towards the toxicity of T-2 toxin. Principle phenomena responsible are the de-epoxidation and de-acetylation activity which occurs in the rumen (Adhikari et al., 2017).

In the rumen fluid HT-2 toxin is formed due to deacetylation of T-2 toxin. HT-2 toxin is further metabolised to T-2 triol. Microorganisms in the rumen fluid have been found to play an active role in metabolising T-2 toxin to less toxic products (Krizova, Rapotin, Pavlok, & Rapotin, 2013). A rapid elimination is exhibited by the main part of Fusarium toxins whereas slower excretion is exhibited by ingested small amounts. Traces of Fusarium toxins could be present in animal tissues (Cavret & Lecoeur, 2006).

OTA could not be detected in any of the samples from New Zealand or China. Bailly & Guerre (2009) have reported the presence of OTA in chicken liver (4.06-7.68 µg/kg) and gizzard (1.89-2.26 µg/kg) samples. (J∅ rgensen, 1998) also has reported that 94.6% of pork kidneys were positive for OTA (> 0.02 ng/g) and a maximum OTA content of 15 ng/g was reported. The absence of OTA observed in the present study could be due to the detoxifying mechanisms of ruminants. Ruminants are known to have a higher tolerance for OTA than non-ruminants due to the presence of rumen protozoans capable of degrading OTA into less toxic metabolites (Abd-Elghany & Sallam, 2015).

Table 4.4 represents the average prevalence levels of mycotoxins (AFB_1, T-2, DON, OTA and ZEA) detected in sheep offal purchased from New Zealand and China.

Table 4.4 Natural incidence of mycotoxin in sheep offal

Country	Offal type	Average mycotoxin prevalence (μg/kg)				
		AFB_1	T-2	DON	OTA	ZEA
New Zealand	Pizzle	2.79±1.32 (5/5)	2.03 (1/5)	ND (0/5)	ND (0/5)	ND (0/5)
	Kidney	6.55±6.09 (5/5)	2.37±1.82 (5/5)	1.85±0.86 (3/5)	ND (0/5)	ND (0/5)
	Heart	0.91±0.53 (5/5)	1.37±0.15 (2/5)	0.71±0.04 (2/5)	ND (0/5)	ND (0/5)
	Testes	1.17±0.93 (5/5)	ND (0/5)	1.616 (1/5)	ND (0/5)	ND (0/5)
	Tripe	1.02±0.94 (4/5)	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)
	Liver	0.88±0.76 (5/5)	ND (0/5)	1.58 (1/5)	ND (0/5)	ND (0/5)
	Tail	11.75±7.59 (5/5)	ND (0/5)	2.98±1.01 (5/5)	ND (0/5)	ND (0/5)
	Skirt	13.77±7.55 (5/5)	3.512 (1/5)	7.03±4.52 (5/5)	ND (0/5)	ND (0/5)
China	Pizzle	0.13±0.05 (2/5)	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)
	Kidney	0.51±0.15(5/5)	ND (0/5)	0.19±0.12 (5/5)	ND (0/5)	ND (0/5)
	Heart	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)
	Testes	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)
	Tripe	0.29 (1/5)	ND (0/5)	0.08 (1/5)	ND (0/5)	ND (0/5)
	Liver	0.83 (1/5)	ND (0/5)	0.28 (1/5)	ND (0/5)	ND (0/5)
	Tail	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)	ND (0/5)
	Skirt	ND (0/5)	0.03±0.01 (2/5)	ND (0/5)	ND (0/5)	ND (0/5)

ND: Not detected

*** Within parentheses are the number of samples positive as a fraction**

OTA is strongly absorbed and metabolized and excreted either via urine or via bile as conjugated metabolites (Yiannikouris & Jouany, 2002).Microorganisms present in the rumen of ruminants are capable of hydrolysing toxic OTA to less toxic OTAα (Yiannikouris & Jouany, 2002). However, in the presence of very high levels of OTA the rumen's detoxification capacity may be exceeded (Yiannikouris & Jouany, 2002). In a study where adult pigs were fed with

feed contaminated with crystalline OTA (200 µg/kg), the liver and kidney were found to have 6.3 ± 1.7 and 9.6 ± 2.7 µg/kg OTA, respectively. OTA was absent in the tissues of the pigs fed with an OTA free diet (control group). This suggests that absence of OTA observed in the offal samples from both countries in the present study maybe due to the absence of OTA in the feed (Dall'Asta et al., 2010). Moreover, low levels of OTA are likely to occur in New Zealand -produced food as result of GAP and GMP practices (The New Zealand Mycotoxin Surveillance Program 06-14 Report series, 2014).

Zearalenone (ZEA) is another mycotoxin produced mainly by fungi belonging to the genus *Fusarium,* in foods and feeds (Martins et al., 2020). Moreover, meat consumption was also found to be positively correlated to the total ZEN levels in urine (Martins et al., 2020). However, in the present study none of the sheep offal samples from New Zealand or China were positive for ZEA. Similar to OTA, ZEA is also strongly metabolized and excreted via bile or urine in ruminants (Yiannikouris & Jouany, 2002). A study from Iran revealed that buffalo liver (68.57%) showed higher ZEA contamination compared to meat (41.42%) and milk (21.42%). The higher incidence of ZEA in the liver maybe due to the hepatic biotransformation pathways (Bailly & Guerre, 2009). In a previous study where male turkeys were fed with a diet containing 0.04 mg/kg neither ZEA nor its metabolites were detected in the breast meat or liver (Dänicke et al., 2007). Sheep are capable of metabolizing ZEA to zearalanone, α-zearalenol, β-zearalenol, α-zearalanol and β-zearalanol (Miles et al., 1996). If present in high levels these will be excreted via urine in the form of glucuronides (Miles et al., 1996).

Deoxynivalenol (DON) is a group B trichothecene which is a threat to crops worldwide and is known cause various diseases in humans and animals (Wang et al., 2014). Trichothecenes can be passed on to meat if the meat producing animal is fed with feed contaminated with trichothecenes. Consumption of such meat could expose humans to tricothecenes though the exposure is minor compared to the direct consumption of contaminated grain products (Seeling et al., 2006; Zou et al., 2012). DON was present in the Kidney (1.85±0.86 µg/kg), heart (0.71±0.04 µg/kg), testes (1.616 µg/kg), liver (1.58 µg/kg), tail (2.98±1.01 µg/kg) and skirt (7.03±4.52 µg/kg) samples from New Zealand. All of the analysed tail and skirt samples were positive for DON. On the contrary, only one sample each of testes and liver were found positive for DON. DON was absent in all of the pizzle and tripe samples. In the rumen of ruminants, DON is converted to less toxic de-epoxy-DON (DOM). DON is known to completely degrade in the rumen and excrete via the renal route (Völkel et al., 2011).

Don was present in kidney (0.19±0.12 μg/kg), tripe (0.08 μg/kg) and liver (0.28 μg/kg) samples purchased in China. All of the kidney samples were positive for DON. DON was absent in pizzle, heart, testes, tail and skirt samples. Kidney is expected to have high DON concentrations as urine is the main mode of excretion (Goyarts, Dänicke, Valenta, & Ueberschär, 2007). This explains the detection of DON in all of the kidney samples. A study from China related to chicken tissues revealed the presence DON in two kidney samples (1.3 and 2.0 μg/kg) and one muscle sample (2.1 μg/kg) (Xu et al., 2014).The DON levels detected in the kidney, tripe and liver samples were lower than the previously reported results. The lower incidence can be explained by the degradation of mycotoxins by microbiota.

Overall, all of the New Zealand offal were positive for AFB_1. The highest and lowest AFB_1 concentrations were present in skirt (13.77±7.55 μg/kg) and liver (0.88±0.76 μg/kg) samples respectively. In the Chinese samples only pizzle, kidney, tripe and liver were positive for AFB_1. The highest concentration was detected in liver (0.83 μg/kg) and the lowest was in pizzle (0.51±0.15 μg/kg). T-2 toxin was only present in the kidney, heart and pizzle samples of New Zealand. The highest and lowest concentrations were present in skirt (3.512 μg/kg) and heart (1.37±0.15 μg/kg) respectively. In the Chinese samples T-2 toxin was present only in skirt samples (0.03±0.01 μg/kg). All offal types from New Zealand were positive for DON except the pizzle and tripe samples. In the Chinese samples, only liver, tripe and kidney were positive for DON. OTA and ZEA were not present in any of the sheep offal from either of the countries.

4.4 Antibacterial effect of chitosan on *S. aureus* and *E. coli* O157:H7

4.4.1 Determination of minimum inhibitory concentrations for *S. aures* and *E. coli* O157:H7

S. aureus and *E. coli* O157:H7 were treated with irradiated and non-irradiated crab chitosan and squid pen chitosan.

S. aureus was expected to show higher sensitivity towards chitosan's antibacterial effect as it is a Gram-positive bacteria, which carries negatively charged teichoic acids on its cell wall that facilitates initial contact with the polycationic chitosan molecules (Raafat et al., 2008). In previous studies, *S. aureus* was found to be more sensitive to crab chitosan than *E. coli* (Metin et al., 2019; Islam et a., 2011). Metin et al. (2019) reported that *S. aureus* (13.5 ± 0.71 mm) exhibited higher inhibition in growth than *E. coli* (12.5 ± 0.71 mm) when treated with 10 mg/ml blue crab chitosan. A similar observation was also reported by Islam et al. (2011) where *S. aureus* (1300 ppm) showed a lower minimal bactericidal concentration than *E. coli* (1400 ppm). In the present study *S. aureus* clearly exhibited a higher sensitivity towards non-irradiated crab chitosan treatment than *E. coli* (Table 4.5). When non-irradiated and irradiated squid pen chitosan were used, similar MIC values were achieved for both bacteria (Table 4.5), and when *E.coli* was treated by irradiated crab chitosan, it was more sensitive than *S. aureus* (Table 4.5). In a study where the antibacterial effect of crab chitosan with different degree of deacetylation (DDA) values was investigated, *E. coli* was found to be more sensitive than *S. aureus* (Huang et al., 2020).

Table 4.5 represents the MIC values obtained for *S. aureus* and *E.coli* O157:H7 when exposed to different treatments of chitosan at different concentrations (0.313, 0.156, 0.078 and 0.039 mg/ml), Ampicillin (64, 32, 16, 8 and 4 µg/ml) and 1% acetic acid (0.063, 0.031, 0.016, 0.008 and 0.004%). Table 4.6 indicates the DDA and Mw values of the crab (non-irradiated and irradiated) and squid pen chitosan (non-irradiated and irradiated) used for the trials.

Table 4.5 Minimum inhibitory concentration (MIC) values of *S. aureus* and *E. coli* O157:H7 exposed to different treatments.

Type of treatment	MIC	
	S. aureus	***E. coli***
Non-irradiated crab chitosan	0.08 mg/ml	0.31 mg/ml
Irradiated crab chitosan	0.63 mg/ml	0.16 mg/ml
Non-irradiated squid pen chitosan	0.31 mg/ml	0.31 mg/ml
Irradiated squid pen chitosan	0.31 mg/ml	0.31 mg/ml
Acetic acid	0.06%	0.06%
Ampicillin	<4 µg/ml	8 µg/ml

* For each trial two replicates were used

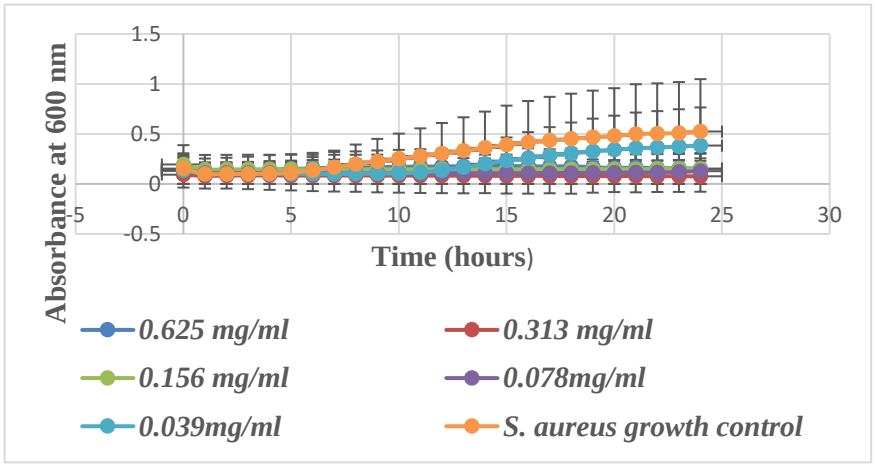
Table 4.6 Degree of deacetylation (DDA) and molecular weights (Mw) of crab and squid pen chitosan (non-irradiated and irradiated).

Type of chitosan	DDA (%)	Mw (kDa)
Non-irradiated crab chitosan	85.00	889.15
Irradiated crab chitosan	85.66	215.22
Non-irradiated squid pen chitosan	72.78	7238.23
Irradiated squid pen chitosan	81.39	502.40

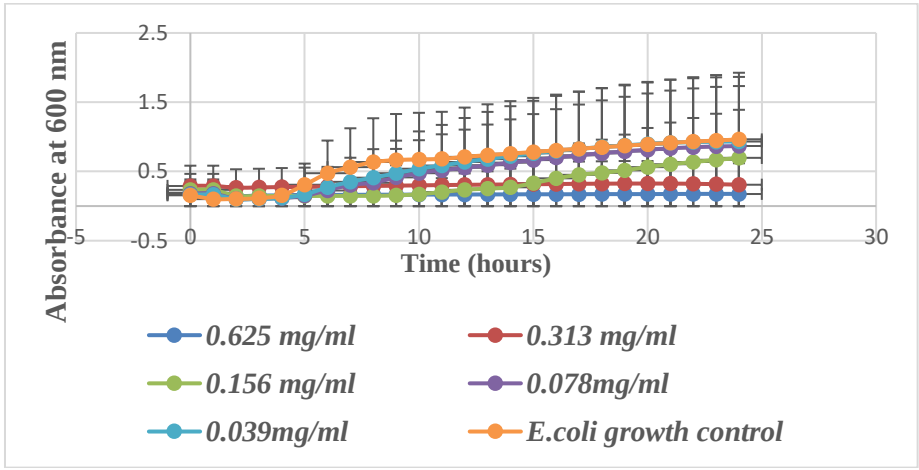
In previous studies, better antibacterial activity was reported by irradiated chitosan than non-irradiated chitosan (Shavandi et al., 2015; Ocloo et al., 2012). Ocloo et al. (2012) reported that non-irradiated crab chitosan at concentrations of 0.02, 0.04 and 0.06% was found to reduce *E. coli* populations by 1-2 logs after 24 to 48 hours of treatment. However, up to 3 logs were obtained at the same concentration using irradiated crab chitosan within 12-48 hours. In the same study, non-irradiated crab chitosan at 0.04% was used to treat *Salmonella paratyphi* and no effect was found after 48 hours but when irradiated crab chitosan at the same concentration was used, 1-2 log reductions were observed after 24-48 hours. In Shavandi et al. (2015)'s study when *E. coli* and *S. aureus* were exposed irradiated crab and squid pen chitosan lower MIC values were observed than when exposed to non-irradiated chitosan. When chitosan is exposed to irradiation, an increase in the degree of deacetylation occurs (Shavandi et al., 2015) which leads to a higher positive charge in chitosan leading to a better interaction with the bacterial cell (Chen et al., 2002).

In the present study the same chitosan used by Shavandi et al. (2015) was used. *E. coli* had a lower MIC value when treated with irradiated crab chitosan than when treated with non-irradiated crab chitosan (Table 4.5) which agrees with previous studies (Shavandi et al., 2015; Ocloo et al., 2012). In contrast, *S. aureus* exhibited a higher MIC when treated with irradiated crab chitosan than with non-irradiated crab chitosan (Table 4.5). Similar MIC values were observed for *S. aureus* and *E. coli* when treated with the non-irradiated and irradiated squid pen chitosan. Overall, in the present study no significant improvement in MIC values were observed for *S. aureus* or *E. coli* when treated with irradiated chitosan compared to non-irradiated chitosan. This may have been due to irradiated chitosan losing its effect during prolonged storage (4 years).

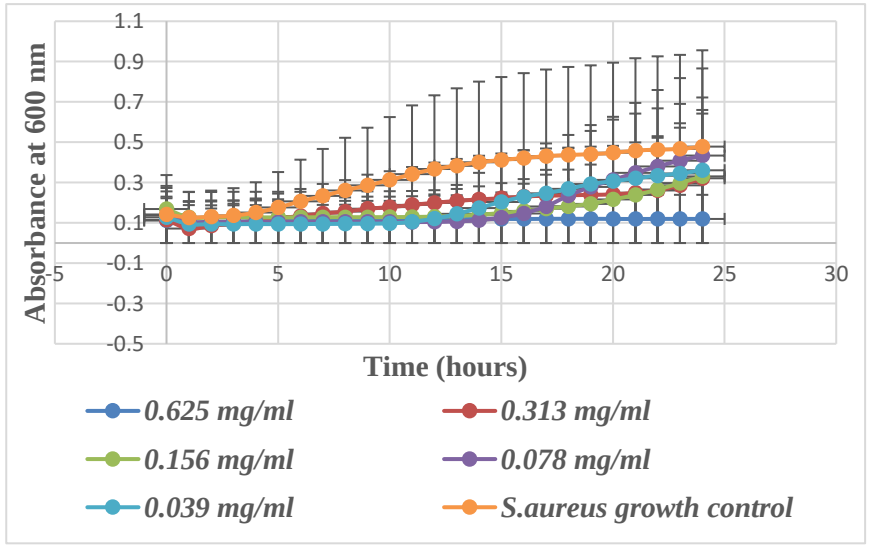
Figures 4.8, 4.9 and 4.10 are graphical representations of the growth curves of *S. aureus* and *E. coli* O157:H7 when treated with the different (non-irradiated and irradiated) crab chitosans, squid pen chitosans, 1% acetic acid and Ampicillin at the respective concentrations.



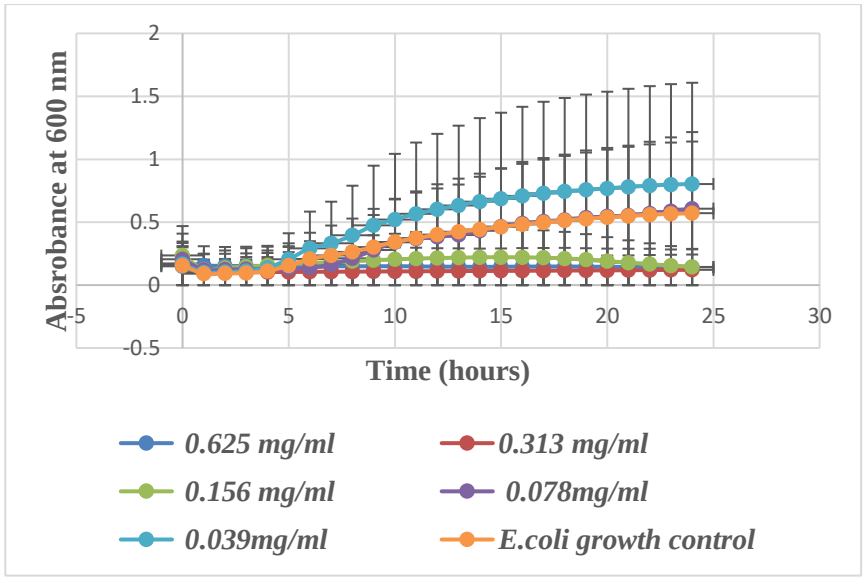
(H)



(I)

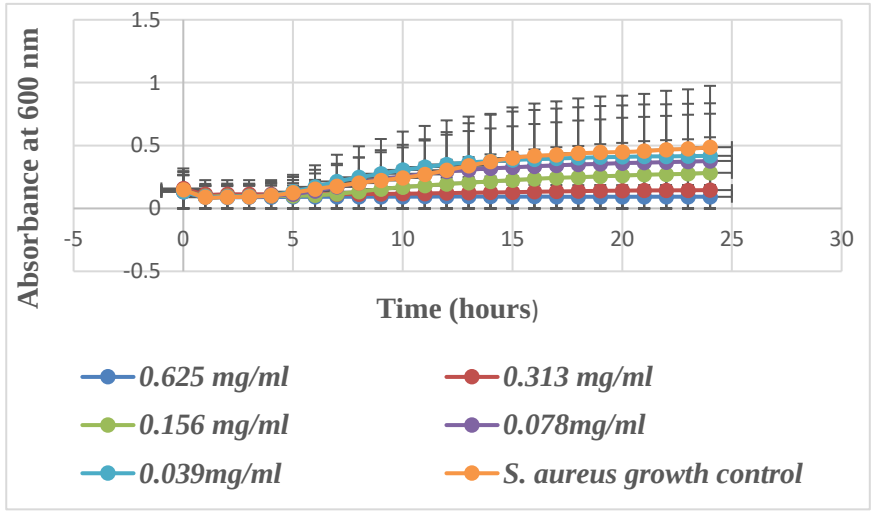


(J)

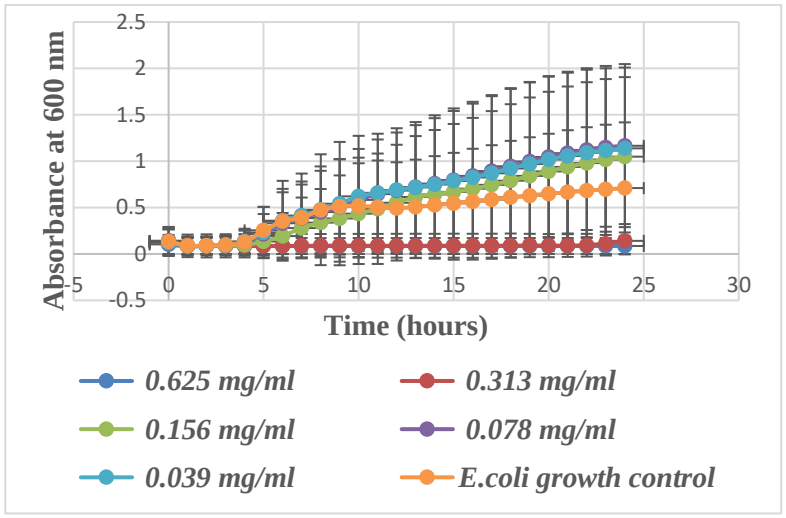


(K)

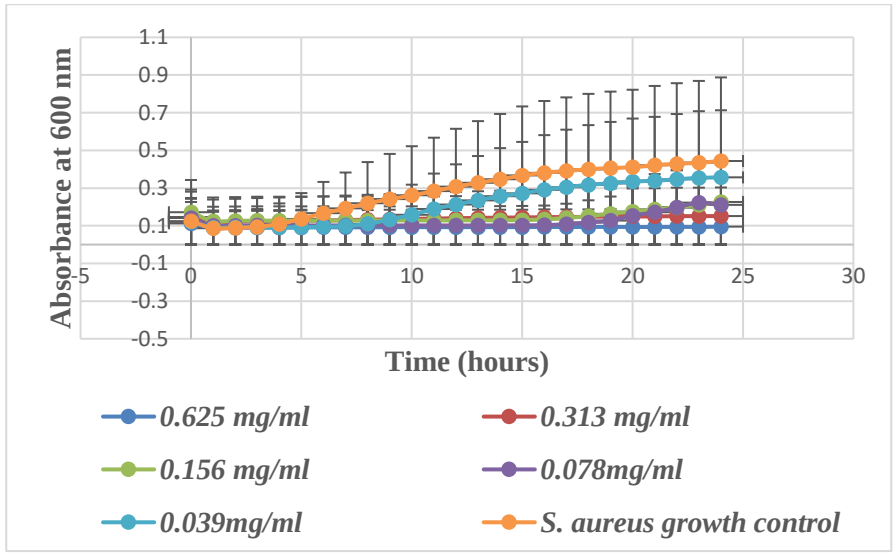
Figure 4.8 Effects of non-irradiated crab chitosan on *S. aureus* (H) and *E. coli* (I) and irradiated crab on *S. aureus* (J) and *E. coli* (K).



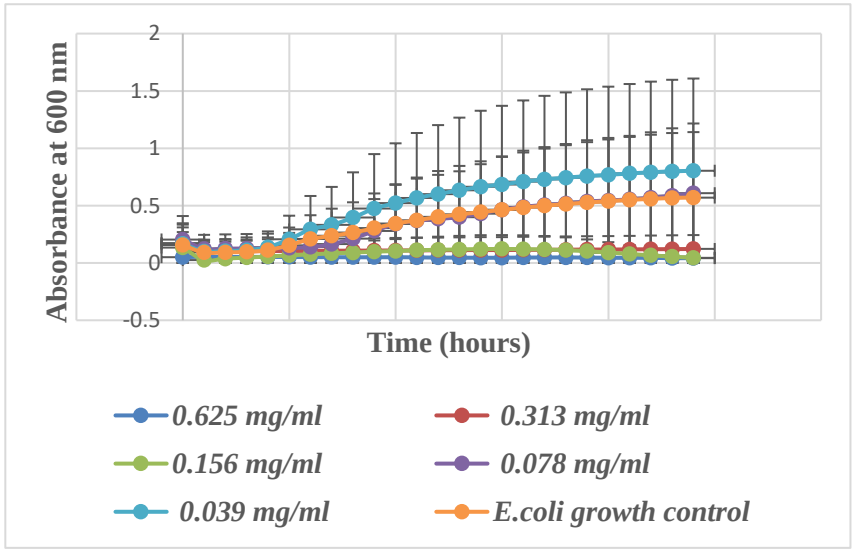
(L)



(M)

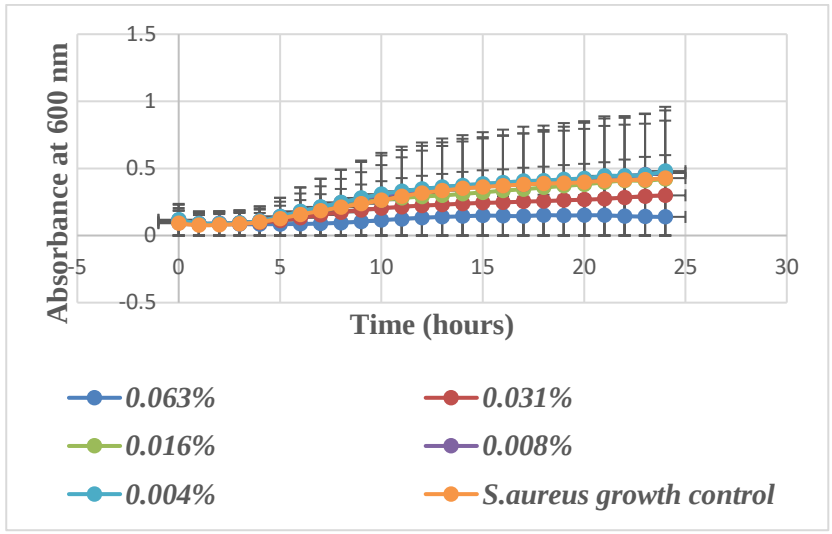


(N)

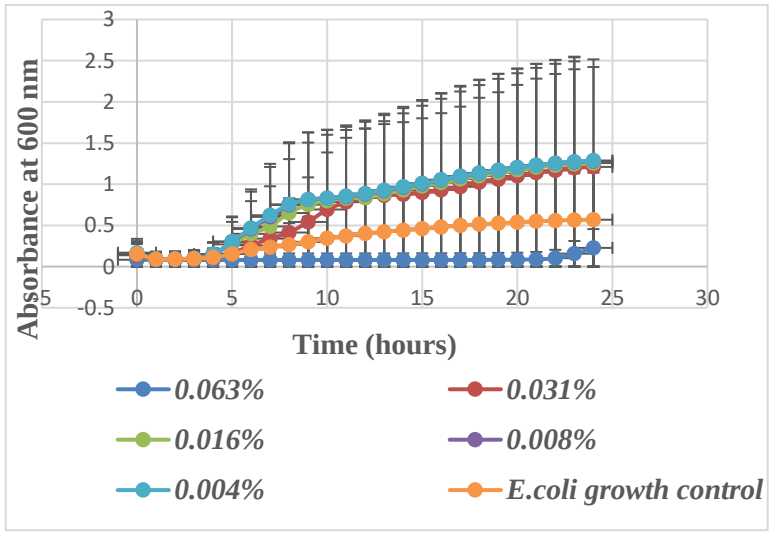


(O)

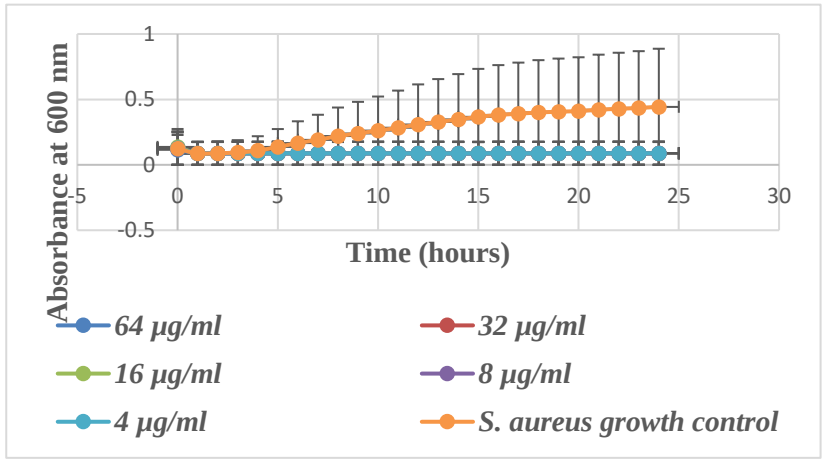
Figure 4.9 Effect of non-irradiated squid pen chitosan on *S. aureus* (L) and *E. coli* (M) and irradiated squid pen chitosan on *S. aureus* (N) and *E. coli* (O).



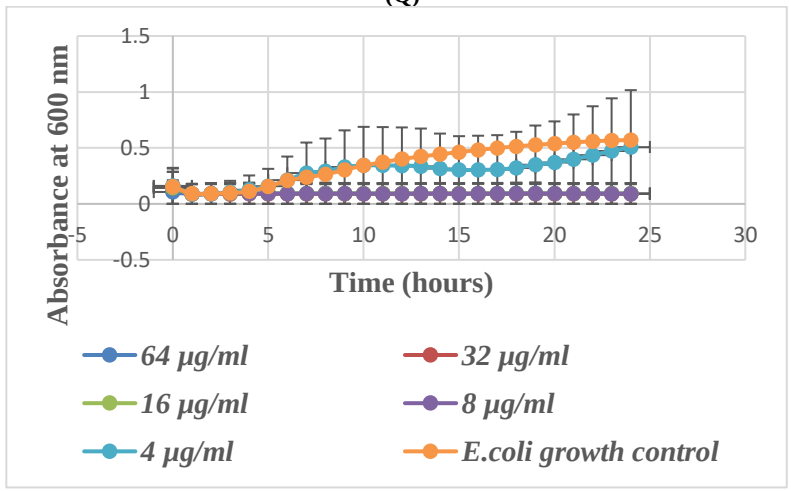
(P)



(Q)



(R)



(S)

Figure 4.10 Effects of acetic acid on *S. aureus* (P) and *E. coli* (Q) and Ampicillin on *S. aureus* (R) and *E. coli* (S).

Zheng and Zhu (2003) in a previous study showed that *S. aureus*, when exposed to chitosan with a range of Mws (<5, 48.5, 72.4, 129, 166 and 305 kDa) showed an increase in inhibition as the molecular weight increased. The possible mechanism suggested for this finding was the chitosan's ability to form a polymer membrane on the cell surface, which will obstruct nutrient entry to the cell. The non-irradiated crab chitosan used in the present study had a higher Mw than the irradiated form (Table 4.6). This may have contributed towards the better antibacterial efficacy of non-irradiated chitosan towards *S. aureus*. Zheng and Zhu (2003) also showed that *E. coli* exhibited better inhibition at lower Mws. This trend is also visible in the present study where *E. coli* exhibits a lower MIC of 0.156 mg/ml when treated with irradiated crab chitosan of lower molecular weight than non-irradiated chitosan. Low Mw chitosan is known to enter the cell via pervasion and adsorb electronegative material within the cell. Later bacterial death occurs as a result of disturbed physiological activities (Zheng and Zhu, 2003).
When *S. aureus* and *E. coli* were treated with non-irradiated and the irradiated squid pen chitosan similar MIC values were obtained (Table 4.5). The irradiated squid pen chitosan had a higher DDA value and is expected to result in better bacterial inhibition as reported by Chen et al. (2007). Chen et al. (2007) reported that *S. aureus* when treated with chitosan with 70% showed 18% inhibition, but when treated with chitosan with 90% DDA showed 35% inhibition. The study further reported that *E. coli* when treated with chitosan of DDA values of 70% and 90% resulted in growth inhibition of 23 and 38% respectively depicting a similar trend.

In the present study in addition to MIC determination the effect of chitosan on the growth of *E. coli* and *S. aureus* was also investigated. The findings depict that an increase in concentrations lead to extended lag phases (Figure 4.8 & 4.9). This agrees with previous studies where the antibacterial activity of chitosan was found to increase with increasing in concentration (Chen et al., 2002).This is in agreement with the findings reported by Chen et al. (2002) where an increased antibacterial activity of chitosan (obtained from shrimp) on *E coli* and *S. aureus* was observed when concentrations were increased (1000, 2500 and 5000 ppm). Zheng and Zhu (2003) reported that when the chitosan concentration was increased from 0.25% to 1.00% complete inhibition was observed for both *E. coli* and *S. aureus* at 1.00% concentration.

S. aureus and *E. coli* had a MIC of 0.06% when treated with 1% acetic acid (Table 4.5). Compared to the results of the present study Shavandi et al. (2015) reported higher MIC values for *E. coli* (0.13%) and *S. aureus* (0.25%) when treated with 1% acetic acid. Arkoun et al. (2017) reported MIC values of 0.05% and 0.15% for *E. coli* and *S. aureus*, respectively. Overall, in the present study the MIC values obtained for *E. coli* and *S. aureus* when treated

with different chitosan solutions were much lower than that observed when treated with 1% acetic acid alone (Table 4.5). The lower MIC values obtained when treated with the chitosan solutions is a result of the synergistic effect between chitosan and acetic acid. Arkoun et al. (2017) reported that when chitosan dissolved in water was used to treat *E. coli* higher minimal bactericidal concentrations were observed than when dissolved in acetic acid suggesting a synergistic effect between acetic acid and chitosan. It is assumed acetic acid results in a physical alteration of the bacterial cell wall. Acetic acid is found to be more effective against Gram-negative bacteria than Gram-positive bacteria due to the absence of murein in the cell wall which makes it more susceptible towards acetic acid unlike Gram-negative bacteria (Ryssel et al., 2008).

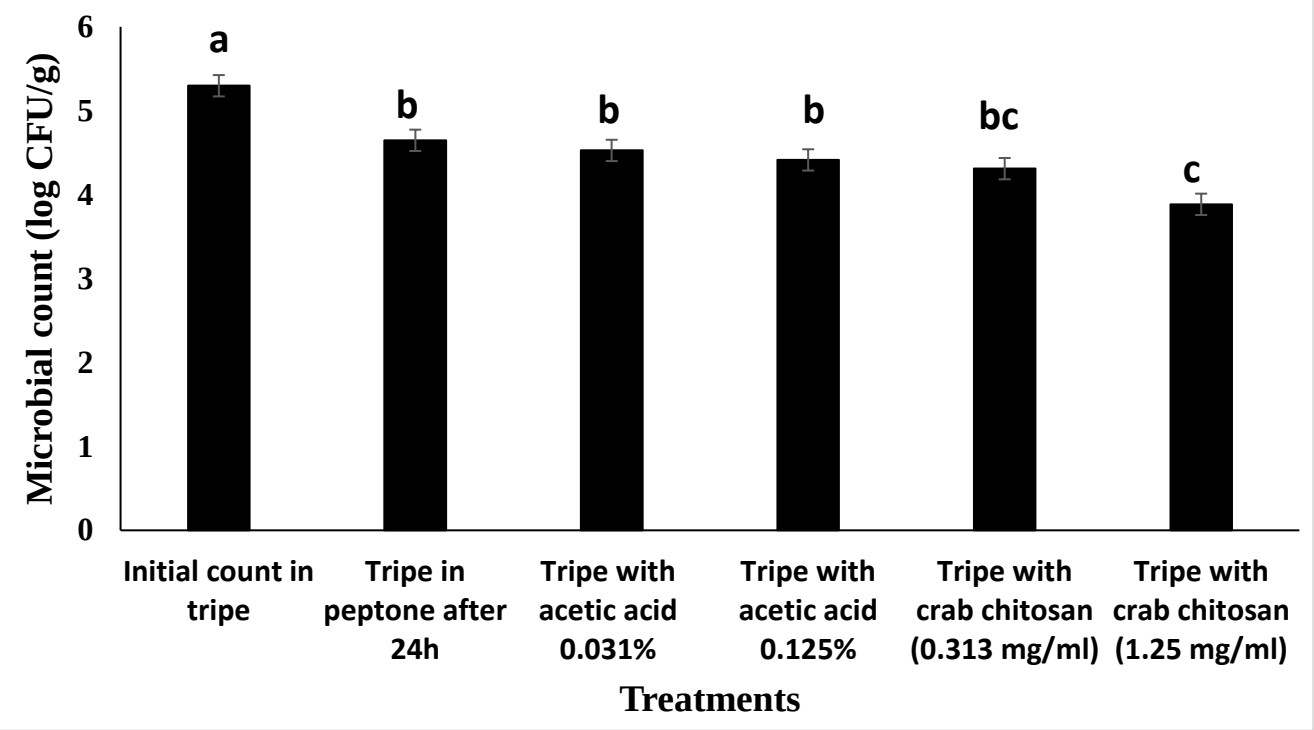
When exposed to Ampicillin, *E. coli* had a MIC of 8 µg/ml whereas, *S. aureus* had a MIC of < 4 µg/ml. These results are expected as Gram-negative bacteria are known to exhibit more resistance towards antibiotics due to the presence of the outer membrane which protects the cell wall (Wulandari et al., 2016). In a study conducted by Muslimin et al.,(2015) when *S. aureus* and *E. coli* isolated from bovine milk were treated with Ampicillin a zone of 42.3 mm was observed for *S. aureus*, but no effect was observed on *E.coli*. This finding clearly indicates the susceptibility of *S. aureus* towards Ampicillin.
Overall, *S. aureus* did not exhibit a high degree of inhibited growth compared to *E. coli* when treated with chitosan as suggested by literature. It is possible that this was due to the differences occurring within the strains used. Hence, performing new trials with a fresh culture or different strain is recommended. Though irradiated chitosan is expected to exhibit better antibacterial activity than non-irradiated chitosan, in the present study a marked difference was not observed between the non-irradiated and irradiated forms of chitosan. It is possible that the chitosan lost its effect during prolonged storage.

4.4.2 Effect of chitosan on tripe inoculated with *S. aureus* and *E. coli* O157:H7

Previous studies have reported chitosan as an effective antibacterial agent against *S. aureus* and *E. coli* O157:H7 (Chen et al., 2002; Shavandi et al., 2015; Zheng & Zhu, 2003). Therefore, these two strains were used to inoculate tripe to further investigate chitosan's antibacterial effect on the two bacteria. Crab chitosan was chosen for the trials due to its high availability and its readily soluble nature in 1% acetic acid. The non-irradiated form was chosen over the irradiated form, as a high level of antibacterial activity was not exhibited by the irradiated form during the MIC trials.

When tripe was inoculated with *E. coli* and treated with peptone water (control) and incubated for 24 hours at 4^0C, a significant reduction ($p<0.05$) in number of *E. coli* compared to the initial count was observed (Figure 4.9).

The initial E. coli count on the tripe samples was 5.30 log CFU/g .The *E. coli* inoculated tripe samples when treated with acetic acid 0.03 and 0.13% (stored at 4^0C for 24 hours) showed *E. coli* counts of 4.53 and 4.41 log CFU/g, respectively which were significantly lower than the initial count ($p<0.05$) (Figure 4.9). However, the reduction in *E. coli* counts observed at the two acetic acid concentrations were not significantly different. Tabbouleh, a traditional Middle Eastern salad when inoculated with *E. coli* O157:H7 and treated with 0.40% and 0.30% acetic acid, a significant reduction was observed when treated with 0.4% and stored at 4^0C for 7 days (Al-Rousan et al., 2018).



***Column bars with different superscripts indicate depict significant difference at $p<0.05$**

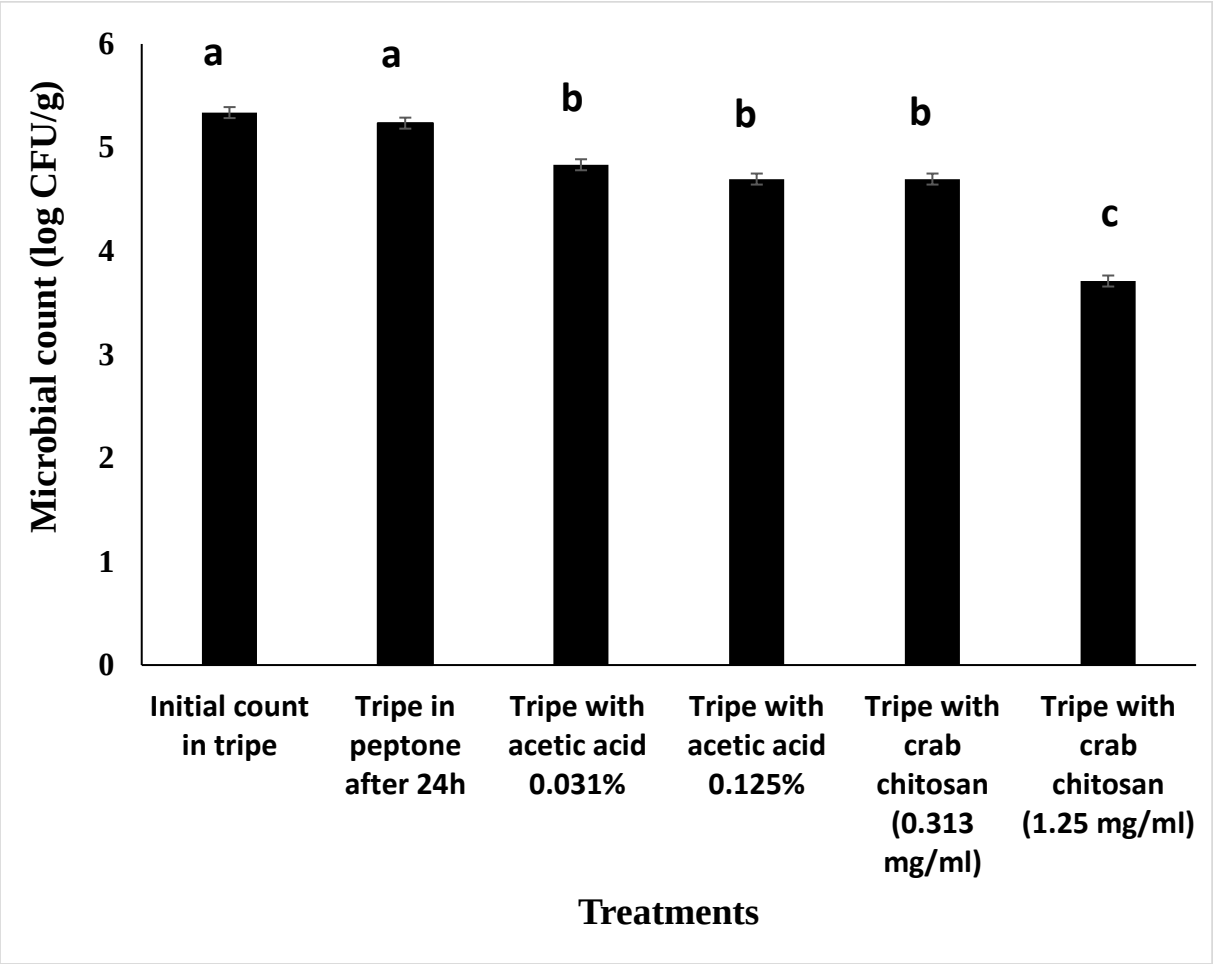
Figure 4.11 Effect of different treatments on tripe inoculated with *E. coli.*

Figure 4.11 represents the different *E. coli* O157:H7 counts present on the tripe samples subjected to different treatments during the investigation of chitosan's ability to effectively decontaminate meat. Figure 4.11 represents the different *S. aureus* counts present on the tripe

samples subjected to different treatments during the investigation of chitosan's ability to effectively decontaminate meat.

E. coli inoculated tripe treated with crab chitosan at 0.31 mg/ml also resulted in a significantly reduced *E. coli* count (4.31 log CFU/g) compared to the initial count ($p<0.05$), (Figure 4.11). Yet, this reduction was not significantly different from the reductions caused by acetic acid (0.03% and 0.13%). This could be understood as 0.31 mg/ml of non-irradiated crab chitosan was the MIC value of *E. coli* (Table 4.5). MIC is the minimum concentration of the crab concentration required to inhibit the bacterial growth and this quantity may be insufficient to significantly reduce *E. coli* count. When the chitosan concentration was increased to 1.25mg/ml a reduction in *E. coli* (3.89 log CFU/g), significantly different to the *E. coli* counts obtained with the acetic acid treatments ($p<0.05$) was achieved (Figure 4.9). Similar to the present study different meat products inoculated with E. coli have depicted inhibited growth when treated with chitosan in previous studies (Incili et al., 2020; Kanatt et al., 2013; Shekarforoush et al., 2015).

In a study by Incili et al. (2020) when 0.4% (w/w) squid chitosan was used to treat meatballs inoculated with *E. coli* O157 : H7 on day zero (4.60 ± 0.60 log CFU/g) a significant reduction in number was observed compared to the control (6.10 ± 0.40 log CFU/g). However, from day four onwards the number of *E. coli* O157 : H7 shows a stable trend (Incili et al., 2020). This may have been due to the bacteriostatic effect exerted by chitosan. Beef inoculated with *E. coli* O157 : H7 treated with a pure chitosan film (stored at room temperature) showed a significant reduction in number on the third day (2.89 ± 0.19 log CFU/g) compared to the control (7.76 ± 0.17 log CFU/g) (Cui et al., 2017). A significant 2 log reduction ($p<0.05$) is exhibited in turkey meat fillets inoculated with *E. coli* O157 : H7 counts (stored at 4^0C) when treated with 2% crab chitosan (w/v) (Vardaka et al., 2016). Kanatt et al. (2013) reported that chicken seekh and mutton seekh kababs were inoculated with *E. coli* JM109 (6 log CFU/ml) and dipped in a solution of 2% commercial shrimp shell chitosan (prepared using 1% acetic acid) a reduction in number by 1-2 log cycles was observed. When the meat samples were stored at 0-3^0C for a period of 12 days the *E. coli* count remained constant. In contrast, the uncoated samples (control) depicted an increase in *E. coli* by about 2 log cycles. These results indicate the antibacterial effect of chitosan. A rather contrasting finding was reported by Shekarforoush et al. (2015) when cured chicken meat was inoculated with *E. coli* O157:H7 (4.55 log CFU/g) and treated with a 2% chitosan solution (prepared with 1% acetic acid and 20 ml of sunflower oil). After 48 hours of storage at 3^0C a non-significant reduction ($p>0.05$) of *E. coli* (4.21 log CFU/g) was observed. The chitosan's DDA and Mw (not mentioned in the study) may have affected antibacterial effect of chitosan. The sunflower oil may also have affected result.



*Column bars with different superscripts indicate depict significant difference at $p<0.05$

Figure 4.12 Effect of different treatments on tripe inoculated with *S. aureus*.

The initial *S. aureus* count in tripe was 5.34 log CFU/g. Tripe placed in peptone (control) at 4^0C after 24 hours was found to have 5.24 log CFU/g which showed no significant difference to the initial count.

Tripe inoculated with *S. aureus* treated with 0.03% acetic acid, 0.13% acetic acid and 0.31 mg/ml crab chitosan showed a significant reduction in S. aureus counts of 4.83, 4.70 and 4.70 log CFU/g respectively compared to the control. When treated with 1.25 mg/ml of crab chitosan a significant reduction in *S. aureus* count of 3.71 log CFU/g was observed compared to the *S. aureus* count in tripe treated with 0.31 mg/ml crab chitosan ($p<0.05$) (Figure 4.12). Similar to the present study different types of meat and sushi inoculated with *S. aureus* have exhibited inhibited growth when treated with chitosan in previous studies (Duran and Khave, 2020; Rachtanapun et al. 2018; Kanatt et al., 2013). Duran and Khave (2020) reported that vacuum packed beef meat inoculated with *S. aureus* (2.00 ± 1.15) treated with 2% chitosan and

stored at 4^0C were completely inhibited after 15 days of storage. Rachtanapun et al. (2018) reported a 1 log reduction in *S. aureus* on sushi rice on day zero when treated with 0.1% crab chitosan. In a study by Kanatt et al. (2013) chicken seekh and mutton seekh kababs were inoculated with *Staphylococcus aureus* ATCC 6538P (6 log CFU/ml) and treated with 2% commercial shrimp chitosan a reduction of about 2-3 log cycles was observed in the meat samples on day zero. In comparison to the uncoated control samples, the chitosan treated samples depicted inhibited bacterial growth stored at 0-3^0C for 12 days.

In the present study overall, *S. aureus* showed a greater reduction (2 log cycles) in number than *E. coli* (1 log cycle) when treated with 1.25 mg/ml of chitosan. A similar finding was reported by Kanatt et al. (2013) where chicken seekh and mutton seekh kababs inoculated with test organisms (6 log CFU/ml) were coated with 2% commercial shrimp chitosan. According to Kanatt et al. (2013) the viable counts of *S. aureus* and *E. coli* reduced by 2-3 and 1-2 log cycles respectively. The higher effectiveness of the chitosan treatment towards Gram-positive bacteria is mainly due to the interaction of a polycation with cell membranes resulting in high membrane permeability which later leads on to the leakage of cell contents such as proteins, ions, enzymes,

Overall, non-irradiated crab chitosan was used to treat sheep tripe samples inoculated with *S. aureus* and *E. coli* O157:H7. The *E. coli* count on tripe was significantly reduced ($p<0.05$) to 4.31 log CFU/g and 3.88 log CFU/g when treated with chitosan at 0.31 mg/ml and 1.25 mg/ml respectively from an initial count of 5.30 log CFU/g. A significant reduction ($p<0.05$) in *E. coli* count was observed only with the 1.25 mg/ml treatment. The *S. aureus* count on the tripe samples was found to be reduced ($p<0.05$) to 4.695 log CFU/g and 3.710 log CFU/g when treated with chitosan at 0.31 mg/ml and 1.25 mg/ml, respectively compared to the initial *S. aureus* count (5.34 log CFU/g).

Chapter 5: Conclusion and recommendations for future work

5.1 Conclusion

The absence of the investigated foodborne pathogens (*C. jejuni*, *Salmonella* spp., *C. perfringens*, *L. monocytogenes coliforms* and the low APC counts in the New Zealand sheep offal samples indicate that the meat was handled under sterile conditions while adhering to HACCP procedures. The absence of the observed pathogenic bacteria in the meat samples may be a result of the successful practice of the Microbiological limit Standard 1.6.1 implemented by Food Standards Australia New Zealand (FSANZ). The absence of the observed pathogenic bacteria in the Chinese offal may be due to the amendments made to the Food Safety Law 2009 with an emphasis on risk-based standards. Though the investigated foodborne pathogens were absent in the Chinese sheep offal samples the comparatively higher coliform counts and APC counts indicate that attention still needs to be paid towards the meat handling practices to ensure high meat quality. However, since limited studies are present evaluating the microbiological quality of different sheep offal in New Zealand and China further research need to be conducted to confirm the safety levels of these meat products.

The metagenomics results indicate that the major phyla present in the offal purchased from both countries were Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Tenericutes and Fusobacteria. However, the level of abundance of the phyla varied between sheep offal samples varied between the two countries. This may have been due to the difference in dietary patterns of the sheep. For instance, the high abundance of *Bacteroidetes* observed in New Zealand offal samples may be due to the presence of a higher proportion of non-fibrous carbohydrates and polysaccharides in the feed. Therefore, analysing the composition of the feed would help to understand the presence of different phyla on the meat samples better. At the species level certain psychrotropic pathogenic bacteria were found to be present on meat samples which could deteriorate the meat quality and also act as opportunistic pathogens. This indicates the need for the investigation of such bacteria on frozen meat offal. In comparison to Chinese offal samples the New Zealand samples exhibited a more diverse microbial population. Therefore, further studies are needed to gain a better understanding of the diverse microbial populations present in New Zealand sheep offal.

AFB_1, T-2 toxin and DON were isolated from sheep offal of both countries. Overall, the New Zealand offal samples depicted a higher affinity towards the detected mycotoxins. However,

OTA and ZEA were not detected in any of the samples. Though ruminants are considered to be less sensitive towards mycotoxins (due to the detoxifying ability), the results of the mycotoxin analysis indicate sheep offal as a potential reservoir of mycotoxins. As the presence of mycotoxins in the observed meat samples may have been due to fungal contamination of the feed or the meat, further studies should be conducted to investigate the source of fungal toxin contamination in New Zealand and Chinese sheep offal.

Though Gram positive bacteria are known to be more susceptible to chitosan's antibacterial effect than Gram negative bacteria, in the present study *S. aureus* (Gram positive) exhibited a higher sensitivity towards chitosan compared to *E. coli* (Gram negative). Furthermore, though previous research suggests that irradiated chitosan has higher antibacterial effect than non-irradiated chitosan, in the current study a significant difference between the antibacterial effects of the irradiated and non-irradiated forms of chitosan were not observed. However, when the same irradiated chitosan used in the present study was used in a previous research it had exhibited better antibacterial effects. This may have been due to the loss of antibacterial properties during prolonged storage. The significant reductions ($p<0.05$) in counts of *S. aureus* and *E. coli* observed in the tripe samples treated with crab chitosan (1.25 mg/ml) suggests that chitosan could serve as a potential natural antibacterial substance which could be used for meat decontamination.

5.2 Recommendations

Several experiments can be performed to extend the further study. Compared to previous work the sample size used for microbiological analysis in the present study was relatively small. Increasing the sample size would be recommended. As fresh offal is mostly used for the preparation of various offal dishes repeating the microbiological analysis using fresh offal samples would be recommended. Performing mycotoxin analysis for the feed fed to the New Zealand and Chinese sheep would help to determine the primary source of contamination. As mycotoxins were found to be present in sheep offal, investigating the presence of mycotoxin producing fungi on sheep offal could be conducted in the future.

According to previous studies, irradiated chitosan depict better antibacterial properties in comparison to non-irradiated chitosan. Since such a trend was not observed in the present study determining the DDA and Mw of the chitosan used in this study would be recommended to determine if changes to DDA and Mw had occurred during the storage period.

The chitosan effect on meat could be further investigated by storing the chitosan treated meat samples at different temperatures (4, 25 and 30^0C) and monitoring the bacterial growth for seven days. This would enable to determine the effect of temperature and storage time on chitosan's antibacterial activity. Treating meat with different concentrations of chitosan to further analyse its antibacterial properties on meat is also suggested. Furthermore, conducting a metagenomic analysis for the chitosan treated meat samples would provide a better understanding of the effect of chitosan towards the meat microbiome.

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